NATIONAL HEART, LUNG, AND BLOOD INSTITUTE ANNUAL REPORT

OCTOBER 1, 1984-SEPTEMBER 30, 1985

INTRAMURAL RESEARCH

THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

ANNUAL REPORT

October 1, 1984 - September 30, 1985

Project Reports - INTRAMURAL RESEARCH

Laboratory of Biochemical Genetics

Laboratory of Biochemistry

Cardiology Branch

Laboratory of Cellular Metabolism

Laboratory of Chemical Pharmacology

Laboratory of Chemistry

Section on Biochemical Pharmacology

Laboratory of Kidney and Electrolyte Metabolism

Surgery Branch

Laboratory of Technical Development

Pulmonary Branch

INTRAMURAL RESEARCH
THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
NUMERICAL INVENTORY OF PROJECTS
October 1, 1984 through September 30, 1985

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Z01 HL 00017-10 LBG
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ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1984 through September 30, 1985

A cDNA clone corresponding to human brain choline acetyltransferase was obtained by screening a λgt11 cDNA library prepared from human basal ganglia poly A^+ RNA with a monospecific antibody directed against affinity-purified choline acetyltransferase from human placenta. One positive clone containing an 1100 bp insert was identified. A 160 kD β-galactosidase-choline acetyltransferase fusion protein synthesized in E. coli was recognized by both anti-β-galactosidase and anti-choline acetyltransferase antibodies on Western blots. The cloned DNA insert hybridizes to one band of RNA from MCIX C human neuroblastoma cells or NS20-Y mouse neuroblastoma cells, that have high choline acetyltransferase activity, but does not hybridize to RNA from N1E-115 neuroblastoma cells that lack choline acetyltransferase activity. The cloned DNA will be characterized further and will be used as a probe to study the mechanism of the switch in gene expression that determines whether peripheral neuroblasts become part of the sympathetic or parasympathetic nervous systems.

Monoclonal antibodies directed against different classes of neurons were obtained by immunizing mice with N18RE-103 cells, a clonal somatic hybrid cell line derived by fusion of 18-day rat embryo retina cells and N18TG-2 mouse neuroblastoma cells. N18RE-103 cells have high tyrosine hydroxylase activity; whereas, tyrosine hydroxylase is not expressed by the parental N18TG-2 cells. Spleen cells from mice immunized with N18RE-103 cells were fused with P3X63 Ag8 mouse myeloma cells; 33 of 374 hybridoma cell lines obtained synthesize antibodies directed against N18RE-103 cells and rat retina cells, but not to non-neural cell lines tested. One of the hybridoma cell lines synthesizes antibody (41C5) that binds to some, but not all, ganglion neuron soma in rat retina, to the outer synaptic layer and the outer limiting membrane of retina, and to some neurons in cerebral cortex and the peripheral nervous system, but not to other tissues tested. A λgt11 cDNA expression library was prepared from poly A^+ RNA from NG108-15 neuroblastoma-glioma hybrid cells and approximately 600,000 recombinant phage plaques were screened with antibody 41C5. One positive plaque was detected. A 140 kD β-galactosidase-41C5 fusion protein, synthesized in E. coli was recognized specifically by antibody 41C5 and by anti β-galactosidase antibody on Western blots. This cDNA clone can be used to study the expression of a gene by neurons with a restricted distribution in the nervous system and by neuroblastoma-hybrid cells in culture.

Elevation of cAMP levels of NG108-15 neuroblastoma-glioma hybrid cells or NS20-Y neuroblastoma cells for 5 or more days shifts relatively undifferentiated cells to a differentiated state with respect to some neural properties and increases the ability of the cells to form synapses. Cytoplasmic poly A^+ RNA was prepared from undifferentiated (D^-) logarithmically dividing NG108-15 and NS20-Y cells and from differentiated (D^+) cells that had been treated for 5 days with 1 mM dibutyryl cAMP. cDNA was synthesized from D^+ poly-A^+ RNA and cloned into pBR322; approximately 400,000 and 800,000 recombinants were obtained in the NG108-15 and NS20-Y cDNA libraries, respectively. Four classes of recombinants were found; (1) colonies with more D^+ ^{32}P-cDNA hybridized than D^- ^{32}P-cDNA, (2) colonies with less D^+ ^{32}P-cDNA hybridized than D^- ^{32}P-cDNA, (3) colonies with approximately equal amounts of D^+ and D^- ^{32}P-cDNA hybridized, and (4) colonies

with little or no D^+ or D^- ^{32}P-cDNA hybridized. These results show that prolonged treatment of NG108-15 or NS20-Y cells with dibutyryl cAMP results in increases in the abundance of some species of poly-A^+ RNA, decreases in the abundance of other species of poly-A^+ RNA, but has no effect on the abundance of most species of mRNA studied. The species of cloned cDNA that are regulated by cAMP will be used as probes to study the mechanisms of regulation of mRNA abundance.

The distribution of the GTP binding protein, α-transducin, was determined in retina sections with a monospecific antibody directed against α-transducin purified from bovine rod outer segments, that cross react with α-transducin from chicken retina. In collaborative studies with Allen Spiegel and coworkers, we find that chicken retina rod photoreceptors have abundant α-transducin; whereas cones have little or no α-transducin that is recognized by the antibody. The results suggest either that the phototransduction mechanism in cones requires relatively few molecules of α-transducin compared to rods, that cones may express a different species of α-transducin than rods that is not detected by the antibody to rod α-transducin, or that the phototransduction mechanism in cones differs from that of rods.

The α, β, and γ protein subunits of voltage-sensitive Ca^{2+} channels were solubilized and purified from rat skeletal muscle T-tubule membrane preparations. Rabbits were immunized with the purified proteins and antibodies were obtained that recognize each subunit of the calcium channel. Antibodies to the β-subunit of the calcium channel were coupled to Sepharose and the β-subunit was purified by affinity column chromatography. A single protein with the expected Mr of 50,000 was eluted from the column. The antibodies will be used to detect the expression of cloned recombinant cDNA for calcium channel proteins in E. coli.

The addition of bradykinin to NG108-15 cells results in a transient, hyperpolarization of cells followed by a prolonged depolarization. Concommitantly, bradykinin also increases cellular levels of inositol-1,4,5-trisphosphate (IP_3), presumably by activation of phosphatidyl-4,5-bisphosphate phosphodiesterase ($PI\text{-}P_2$ PDE), and increases the rate of secretion of acetylcholine from NG108-15 cells. Injection of IP_3 or Ca^{2+} into the cytoplasm of NG108-15 cells results in cell hyperpolarization followed by depolarization, similar to the effects elicited by extracellular bradykinin. The results suggest that the hyperpolarizing phase of the cells' response to bradykinin is due to elevation of intracellular IP_3, which stimulates the release of calcium ions from internal storage sites into the cytoplasm, thereby activating calcium-dependent K^+ channels. The depolarizing phase of the cell response to bradykinin is due largely to a decrease in the rate of K^+ efflux from cells due to inhibition of M channels and, to a lesser extent, to activation of calcium-dependent cation channels and calcium channels. Incubation of NG108-15 cells with pertussis toxin inhibits bradykinin-dependent cell hyperpolarization. Collaborative studies with Werner Klee show that bradykinin stimulates low K_m GTPase activity in NG108-15 membrane preparations, but not in membranes prepared from NG108-15 cells treated with pertussis toxin. Incubation of NG108-15 membranes from cells that had been treated with pertussis toxin with a mixture of purified N_o and N_i (>95% pure) from bovine brain restores bradykinin-dependent GTPase activity. Bradykinin also inhibits basal adenylate cyclase activity in NG108-15 hommogenates. These results show that

[bradykin·receptor] complexes interact with N_o or Ni and suggest that N_o and/or N_i may couple bradykinin activation of receptors with activation of low K_m GTPase and PI-P_2 phosphodiesterase with inhibition of adenylate cyclase.

Mice were immunized with N18RE-103, NME-1, or NCE-9AK cells, which were derived by fusion of N18TG-2 mouse neuroblastoma cells with retina cells from rat, mouse, or Chinese hamster embryos, respectively, or with rat striated muscle cell membranes. Fusion of spleen cells from immunized mice with P3X63Ag8 mouse myeloma cells yielded approximately 1200 hybridoma cell lines; 146 of the cell lines synthesize antibodies directed against the immunogens. Ten of 50 monoclonal antibodies tested were found to affect miniature endplate potential frequencies (MEPPS) at synapses between NG108-15 or NBR10-A neuroblastoma hybrid cells and rat myotubes. Four of the antibodies increased, and 6 decreased MEPPS, and 40 antibodies had little or no affect on MEPPS. Two of the antibodies that increased MEPPS, 37E12 and 55F1, and one antibody that decreased MEPPS, P4C11, bound to multiple bands of NBr10-A, or NG108-15 protein on Western blots. These antibodies also bound to neutral glycolipids of the neuroblastoma hybrid cells that were fractionated by DEAE-Sepharose column chromotography. These results show that 3 antibodies that affect MEPPS recognize different sets of molecules of neuroblastoma hybrid cells and suggest that the antigenic sites may be oligosaccharide moieties of glycolipids and glycoproteins.

The regulation of the gene coding for preproenkephalin (ppEnk), the precursor of the opioid peptides methionine- and leucine-enkephalin, is under investigation. A full-length rat ppEnk cDNA clone, constructed and characterized by us last year, was used as a sensitive hybridization probe for ppEnk mRNA in RNA preparations for clonal cell lines of neuronal origin.

NG108-155 mouse neuroblastoma x rat glioma hybrid cells contain small amounts of rat ppEnk mRNA, 20-120 fg/ug RNA by Northern blot analysis. Treatment of NG108-15 cells with glucocorticoids such as dexamethasone elevated the ppEnk mRNA abundance to 3 and 9 times the control at 24 h and 8 days, respectively. Treatment of the cells with 8-Br-cAMP or activators of adenylate cyclase such as forskolin had little or no effect on the ppEnk mRNA abundance. However, treatment with both glucocorticoid and either 8-Br-cAMP or forskolin markedly elevated the ppEnk mRNA abundance to 5-8 times and 30 times the control at 24 h and 8 days, respectively. These increases were blocked by actinomycin D, an inhibitor of transcription. The Met-enkephalin content of the cells was increased in parallel with the mRNA abundance. These results demonstrate that the transcription of the rat ppEnk gene is positively regulated by the synergistic interaction of glucocorticoids and cAMP.

C6 and C6BU-1 rat glioma cells contain a much higher abundance of ppEnk mRNA (3-6 pg/µg RNA) but lower enkephalin content than NG108-15 cells. C6 ppEnk mRNA is markedly but transiently elevated by the combination of glucocorticoids and compounds that elevate cAMP, e.g. beta-adrenergic receptor agonists such as norepinephrine and isoproterenol, which active C6 adenylate cyclase. Glucocorticoids alone were ineffective, while cAMP elevation alone had a slight effect. These results suggest that the ppEnk gene may be expressed in glial cells or their developmental precursors.

Exposure of myotubes to embryonic brain extract results in an increase in the aggregation of nicotinic acetylcholine receptors. After 24 hours of

incubation, the aggregates are larger and more stable at 38°C or in the presence of sodium azide than aggregates formed during the first 4 hours of incubation. The maturation of acetylcholine receptor aggregates resembles changes that occur in the first few days after the formation of acetylcholine receptor aggregates and to developing neuromuscular junction in vivo. Immunofluorescence staining of cryostat sections and cell surface fragments prepared from embryonic brain extract-treated myotubes showed that vinculin and α-actinin concentrations are high at receptor-rich regions of the myotube cell surface. Receptor-rich cell surface fragments also were shown to be stained intensely with the monoclonal antibody against actin.

Protein was extracted from the extracellular matrix of the Torpedo electric organ and was shown to induce extensive acetylcholine receptor aggregation on cultured myotubes with an initial time course similar to that obtained with embryonic brain extract. The receptor aggregation protein was purified approximately 115-fold. The receptor aggregates induced by Torpedo protein were smaller and more numerous than those induced by embryonic brain extract.

Adenylate cyclase activity in intact E. coli cells or in toluene-treated cells can be inhibited by glucose while the activity in broken cell preparations is not inhibited by glucose. Adenylate cyclase activity in the permeabilized but not in broken cells is stimulated somewhat specifically and additively by potassium and phospate. Kinetic studies show sigmoid substrate-velocity curves for the tolune-treated cells but hyperbolic curves for the broken cells. The stimulatory effects of potassium and phosphate on adenylate cyclase activity in tolune-treated cells are associated with increases in the Vmax and Km for ATP. While the enzyme activity in tolune-treated cells shows a preference for magnesium over manganese ions, the reverse is observed in broken cells. Stimulation of adenylate cyclase activity in toluene-treated cells requires the presence of the proteins of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The PTS proteins can be phosphorylated in a phosphoenolpyruvate-dependent reaction. The stimulatory effects of ions will not occur if the PTS proteins are not phosphorylated. Since potassiu;m phosphate stimulates both adenylate cyclase and PTS activities in tolune-treated cells, it is proposed that the effect of potassium phosphate on adenylate cyclase activity is mediated through an effect on the PTS. A model for dual regulation by glucose of adenylate cyclase activity was proposed that involves regulation of both the condition of PTS proteins as well as the cellular concentration of phosphate.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00009-11 LBG

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cell Recognition and Synapse Formation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Marshall Nirenberg, Chief, LBG, NHLBI
David Trisler, Staff Fellow, LBG, NHLBI
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Karl Krueger, Staff Fellow, LBG, NHLBI
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Benjamin Amaladoss, Visiting Fellow, LBG, NHLBI
Koh Yano, Visiting Fellow, LBG, NHLBI

COOPERATING UNITS *(if any)*

William Strauss, Staff Fellow, LDN, NICHD
Victor Ginsberg, Biochemist, LBP, NIADDK
Allen Spiegel, Chief, MDB, NIADDK
Werner Klee, Section Chief, LMB, NIMH

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section of Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
12	10	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Species of cDNA corresponding to choline acetyltransferase and protein 41C5 were cloned; the expression of genes for both species of protein are regulated in neurons during development. In addition, cloned cDNA recombinants were obtained for species of mRNA that are positively or negatively regulated by cyclic AMP in neuroblastoma or hybrid cells.

A monospecific antibody directed against α-transducin was used to determine the distribution of α-transducin in chicken retina. α-Transducin was found to be abundant in rod photoreceptors, but was not detected in cones. These results suggest that rods and cones either express different species of α-transducin or have different mechanisms of phototransduction.

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5

Project Description:

Objectives:

Our objective is to discover basic mechanisms that regulate the expression of genes during cell differentiation.

Major Findings:

A cDNA clone corresponding to human brain choline acetyltransferase was obtained by screening a λgt11 cDNA library prepared from human basal ganglia poly A^+ RNA with a monospecific antibody directed against affinity-purified choline acetyltransferase from human placenta. One positive clone containing an 1100 bp insert was identified. A 160 kD β-galactosidase-choline acetyltransferase fusion protein synthesized in E. coli was recognized by both anti-β-galactosidase and anti-choline acetyltransferase antibodies on Western blots. The cloned DNA insert hybridizes to one band of RNA from MCIX C human neuroblastoma cells or NS20-Y mouse neuroblastoma cells that have high choline acetyltransferase activity, but does not hybridize to RNA from N1E-115 neuroblastoma cells that lack choline acetyltransferase activity. The cloned DNA will be characterized further and will be used as a probe to study the mechanism of the switch in gene expression that determines whether peripheral neuroblasts become part of the sympathetic or parasympathetic nervous systems.

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The distribution of the GTP binding protein α-Transducin was determined in retina sections with a monospecific antibody directed against α-Transducin purified from bovine rod outer segments, that cross react with α-Transducin from chicken retina. In collaborative studies with Allen Spiegel and coworkers, we find that chicken retina rod photoreceptors have abundant α-Transducin; whereas cones have little or no α-Transducin that is recognized by the antibody. The results suggest either that the phototransduction mechanism in cones requires relatively few molecules of α-Transducin compared to rods, that cones may express a different species of α-Transducin than rods that is not detected by the antibody to rod α-Transducin, or that the phototransduction mechanism in cones differs from that of rods.

The α, β, and γ protein subunits of voltage-sensitive Ca^{2+} channels (Mr = 135,000, 50,000, and 32,000, respectively) were solubilized and purified from rat skeletal muscle T-tubule membrane preparations. Rabbits were immunized with the purified proteins and antibodies were obtained that recognize each subunit of the calcium channel. Antibodies to the β-subunit of the calcium channel were coupled to Sepharose and the β-subunit was purified by affinity column chromatography. A single protein with the expected Mr of 50,000 was eluted from the column. The antibodies will be used to detect the expression of cloned recombinant cDNA for calcium channel proteins in E. coli.

The addition of bradykinin to NG108-15 cells results in a transient, hyperpolarization of cells followed by a prolonged depolarization. Concommitantly, bradykinin also increases the cellular levels of inositol-1,4,5-trisphosphate (IP_3), presumably by activation of phosphatidyl-4,5-bisphosphate phosphodiesterase ($PI\text{-}P_2$ PDE), and increases the rate of secretion of acetylcholine from NG108-15 cells. Injection of IP_3 or Ca^{2+} into the cytoplasm of NG108-15 cells results in cell hyperpolarization followed by depolarization, similar to the effects elicited by extracellular bradykinin. The results suggest that the hyperpolarizing phase of the cells' response to bradykinin is due to elevation of intracellular IP_3, which stimulates the release of calcium ions from internal storage sites into the cytoplasm, thereby activating calcium-dependent K^+ channels. The depolarizing phase of the cell response to bradykinin is due largely to a decrease in the rate of K^+ efflux from cells due to inhibition of M channels and, to a lesser

extent, to activation of calcium-dependent cation channels and calcium channels. Incubation of NG108-15 cells with pertussis toxin inhibits bradykinin-dependent cell hyperpolarization. Collaborative studies with Werner Klee show that bradykinin stimulates low K_m GTPase activity in NG108-15 membrane preparations, but not in membranes prepared from NG108-15 cells treated with pertussis toxin. Incubation of NG108-15 membranes from cells that had been treated with pertussis toxin with a mixture of purified N_o and N_i (>95% pure) from bovine brain restores bradykinin-dependent GTPase activity. Bradykinin also inhibits basal adenylate cyclase activity in NG108-15 hommogenates. These results show that [bradykin·receptor] complexes interact with N_o or Ni and suggest that N_o and/or N_i may couple bradykinin activation of receptors with activation of low K_m GTPase and PI-P_2 phosphodiesterase with inhibition of adenylate cyclase.

Mice were immunized with N18RE-103, NME-1, or NCE-9AK cells, which were derived by fusion of N18TG-2 mouse neuroblastoma cells with retina cells from rat, mouse, or Chinese hamster embryos, respectively, or with rat striated muscle cell membranes. Fusion of spleen cells from immunized mice with P3X63Ag8 mouse myeloma cells yielded approximately 1200 hybridoma cell lines; 146 of the cell lines synthesize antibodies directed against the immunogens. Ten of 50 monoclonal antibodies tested were found to affect miniature endplate potential frequencies (MEPPS) at synapses between NG108-15 or NBR10-A neuroblastoma hybrid cells and rat myotubes. Four of the antibodies increased, and 6 decreased MEPPS, and 40 antibodies had little or no affect on MEPPS. Two of the antibodies that increased MEPPS, 37E12 and 55F1, and one antibody that decreased MEPPS, P4C11, bound to multiple bands of NBr10-A, or NG108-15 protein on Western blots. These antibodies also bound to neutral glycolipids of the neuroblastoma hybrid cells that were fractionated by DEAE-Sepharose column chromotography. These results show that 3 antibodies that affect MEPPS recognize different sets of molecules of neuroblastoma hybrid cells and suggest that the antigenic sites may be oligosaccharide moieties of glycolipids and glycoproteins.

Significance to Biomedical Research:

New information was obtained concerning synaptogenesis and gene expression in the nervous system.

Publications:

1. Nirenberg, M., Krueger, K., Rotter, A., Wilson, S., and Higashida, H.: Regulation of Synapse Formation by Cyclic AMP. In: The Symposium Of The International Society for Developmental Neurosciences, pp. 15-16, 1984.

2. Fredman, P., Magnani, J.L., Grunwald, G.B., Trisler, G.D., Nirenberg, M., Ginsburg, V.: Developmental Regulation of Ganglioside Antigens of Antibodies A2B5 and 18B8 in Chicken Embryo Brains and Retinas. In: Cellular And Pathological Aspects of Glycoconjugate Metabolism. H. Dreyfus, ed. Plenum Press, New York. (In Press).

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5. Strauss, W.L. and Nirenberg, M.: Inhibition of Choline Acetyltransferase by Monoclonal Antibodies. The Journal of Neuroscience 1, 175-180 (1985).

6. Grunwald, G.B., Fredman, P., Magnani, J.L., Trisler, D., Ginsburg, V., and Nirenberg, M.: Monoclonal Antibody 18B8 Detects Gangliosides Associated with Neuronal Differentiation and Synapse Formation. Proc. Natl. Acad. Sci., USA 82, 4008-4012 (1985).

7. Grunwald, G.B., Klein, R., Simmonds, M.A., and Kornguth, S.: Autoimmune Basis for Visual Paraneoplastic Syndrome in Patients with Small Cell Lung Carcinoma. Lancet, 658-661 (1985).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL00017-10 LBG

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Acetylcholine Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Mathew P. Daniels, Research Biologist, LBG-NHLBI
Jacqueline E. Krikorian, Guest Worker, LBG-NHLBI

COOPERATING UNITS *(if any)*

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Robert Bloch, U. of Maryland, Medical School, Physiology Dept.
Ralph Nitkin, Stanford U. Medical School, Neurobiology Dept.

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	2	1.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our aim is to study the organization of neurotransmitter receptors on nerve and muscle cells in relationship to the development and function of synapses. Our recent work has focused upon the factors, extinsic and intrinsic to the developing skeletal muscle fiber, which regulate the distribution of nicotinic acetylcholine receptors. Acetylcholine receptor aggregation is induced on cultured myotubes by neuronal factors, and this system is used to study the mechanisms of receptor aggregation, as well as the stabilization or elimination of aggregates which occur in developing neuromuscular junctions. Our major findings in the past year are as follows: 1) Newly-formed receptor aggregates are reorganized in the continued presence of embryonic brain extract (receptor aggregation factor) to form a smaller number of larger aggregates. These aggregates are twice as resistant to disassembly by temperature elevation to 38° or exposure to sodium azide. This maturation of receptor aggregates is analogous to changes which occur during neuromuscular junction formation. 2) Immunofluorescence staining shows that newly-formed receptor aggregrates are associated with concentrations of the cytoskeletal proteins vinculin and α-actinin, which have been implicated in membrane-actin interactions. 3) Crude as well as highly-purified factors from the extracellular matrix of Torpedo electric organ induce rapid formation of receptor aggregates in our system, but the aggregation pattern differs from that induced by embryonic brain extract.

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Project description:

Objectives:

Our aim has been to study the organization of neurotransmitter receptors on nerve and muscle cells in relationship to the development and function of synapses. Our recent work has focused on factors extrinsic and intrinsic to the developing muscle fiber, which regulate the distribution of nicotinic acetylcholine (ACh) receptors. In particular, we have been investigating the induction of ACh receptor aggregation on muscle cells in culture, by soluble macromolecules from neurons. This system serves as a model in which to study the mechanisms of the aggregation of ACh receptors which occurs at the developing neuromuscular synapse, and the mechanisms of stabilization or elimination of receptor aggregates as synapses mature, are remodeled, or are eliminated.

Methods:

We stain rat skeletal myotubes grown in monolayer culture with rhodamine-labeled α-bungarotoxin (α-BT) in order to visualize ACh receptor sites with the fluorescence microscope. Sequential observations of ACh receptor distribution are made by use of a video image intensification system.

The distribution of basement membrane and cytoskeletal proteins in the cultures is determined by indirect immunofluorescence, using antisera, affinity purified antibodies, and monoclonal antibodies against purified proteins. The staining is done on cryostat sections of pelleted cultures and on cell surface fragments from the cultures.

ACh receptor aggregating material is routinely prepared from extracts of fetal pig brain. We are also using crude and highly purified preparations from the extracellular matrix of Torpedo electric organ.

Major Findings:

Recent Background

We have previously shown that: a) ACh receptor aggregates form maximally in 4-6 hours of the exposure of myotubes to embryonic brain extract. b) The precursors of the receptor aggregates are clouds of punctate microaggregates. Microaggregate clouds appear to be less stable than aggregates, and electron microscopy shows them to be poor in the dense cytoskeletal specializations and basal lamina associated with aggregates. However, miacroaggregates have other, more subtle specializations in common with the aggregates. c) Both the formation and stability of receptor aggregates are sensitive to temperature and are energy dependant (sensitive to sodium azide). At 18-23°, microaggregates accumulate but do not become aggregates. Aggregates are disassembled slowly at 36°, but rapidly (and reversibly) at 38° or in the presence of sodium azide.

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Advances made in the past year:

1. Maturation of ACh receptor aggregates - When exposure of myotubes to embryonic brain extract is continued for up to 20 hours after the initial 4 hour period, the 4 hr aggregates average (9 um long) are reorganized to form a smaller number of larger (average 27 um long) oval-shaped aggregates. These 24 hour aggregates are more stable than the 4 hr aggregates, persisting at least twice as long at 38°, or in the presence of sodium azide. This maturation to form more compact, more stable aggregates, resembles changes which occur in the first 2 days after the formation of ACh receptor aggregates at the developing neuromuscular junction in vivo.

2. Cytoskeletal components of ACh receptor aggregates - Immunofluorescence staining on cryostat sections and cell surface fragments prepared from embryonic brain extract-treated myotubes has revealed a concentration of vinculin and α-actinin at the receptor-rich regions of the cell surface. These proteins have been implicated in interactions between the plasma membrane and actin, and in the crosslinking of actin filaments. The receptor-rich cell surface fragments are also stained intensely with a monoclonal antibody against actin.

   ACh receptor aggregation activity from Torpedo electric organ - We have shown that a relatively crude (specific activity 660 units/mg protein) low pH extract from the extracellular matrix of the Torpedo electric organ (an ACh receptor-rich tissue consisting of a battery of specialized neuromuscular junctions) as well as a highly purified protein fraction from the same extract (specific activity 77,000 units/mg protein), can induce extensive ACh receptor aggregation with an initial time course similar to that obtained with embryonic brain extract. However, the appearance of aggregates induced on the surface of myotubes by the Torpedo factors is different from that obtained with embryonic brain extract, the former aggregates being large and 'patchy', the latter being smaller, but more numerous.

Significance to Biomedical Research:

An understanding of the control mechanisms involved in the organization of neurotransmitter receptors at the developing synapse is of clear importance in any attempt to understand the role of neurotransmitters and their receptors in the function and development of the nervous system. Our studies on the interactions between neuronal factors, extracellular matrix components, and cytoskeletal structures may lead to a better understanding of the mechanisms whereby neurons control or modulate the distribution of receptors on muscle fibers and on other neurons, during synapse development and after.

Proposed Course:

1. We will extend our investigation of the maturation of newly-formed ACh receptor aggregates by studying: a) Changes in stability based on the use of additional disruptive treatments, such as incubation with low Ca^{++} medium. b) Changes in receptor site density. c) Changes in associated ultrastructure at the cell surface.

2. We will continue our immunocytochemical studies on the cytoskeletal components of the receptor aggregates and microaggregate clouds, both on the light microscopic and electron microscopic levels. We also plan to initiate a biochemical and immunological study on the composition of ACh receptor-rich cell surface fragments from embryonic brain extract-treated myotubes.

3. If recruitment of a third postdoctoral fellow is successful, we will initiate a study on the role of proteolysis in the disassembly of ACh receptor aggregates.

4. We will continue to compare the activities of the embryonic brain extract and the purified Torpedo factor, with reference to the observed stages of aggregate formation and their sensitivity to temperature and azide.

Publications:

Busis, N.A., Daniels, M.P., Bauer, H.C., Pudimat, P.A., Sonderegger, P., Schaffner, A.E. and Nirenberg, M.: Three cholinergic neuroblastoma hybrid cell lines that form few synapses on myotubes are deficient in acetylcholine receptor aggregation molecules and large dense-core vesicles. Brain Res. 324: 201-210, 1984.

Bauer, H.C., Hasegawa, S., Sonderegger, P., Daniels, M.P., and Pudimat, P.: Specificity of neuronal factors which aggregate acetylcholine receptors on cultured myotubes. Exp. Cell Res. 157: 288-292, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL 00018-08 LBG

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of the biosynthesis of the opioid peptides and other neuropeptides

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Steven L. Sabol, M.D., Ph.D., Medical Officer (Research), LBG, NHLBI
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COOPERATING UNITS *(if any)*
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Hsiu-Ying Yang, Ph.D., Section Chief, Lab. Preclinical Pharmacology, NIMH

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The regulation of the gene coding for preproenkephalin (ppEnk), the precursor of the opioid peptides methionine- and leucine-enkephalin, is under investigation. A full-length rat ppEnk cDNA clone, constructed and characterized by us last year, was used as a sensitive hybridization probe for ppEnk mRNA in RNA preparations for clonal cell lines of neuronal origin.

NG108-15 mouse neuroblastoma x rat glioma hybrid cells contain small amounts of rat ppEnk mRNA, 20-120 fg/ug RNA by Northern blot analysis. Treatment of NG108-15 cells with glucocorticoids such as dexamethasone elevated the ppEnk mRNA abundance to 3 and 9 times the control at 24 h and 8 days, respectively. Treatment of the cells with 8-Br-cAMP or activators of adenyate cyclase such as forskolin had little or no effect on the ppEnk mRNA abundance. However, treatment with both glucocorticoid and either 8-Br-cAMP or forskolin markedly elevated the ppEnk mRNA abundance to 5-8 times and 30 times the control at 24 h and 8 days, respectively. These increases were blocked by actinomycin D, an inhibitor of transcription. The Met-enkephalin content of the cells was increased in parallel with the mRNA abundance. These results demonstrate that the transcription of the rat ppEnk gene is positively regulated by the synergistic interaction of glucocorticoids and cAMP.

C6 and C6-BU1 rat glioma cells contain a much higher abundance of ppEnk mRNA (3-6 pg/μg RNA) but lower enkephalin content than NG108-15 cells. C6 ppEnk mRNA is markedly but transiently elevated by the combination of glucocorticoids and compounds that elevate cAMP, e.g. beta-adrenergic receptor agonists such as norepinephrine and isoproterenol, which activate C6 adenylate cyclase. Glucocorticoids alone were ineffective, while cAMP elevation alone had a slight effect. These results suggest that the ppEnk gene may be expressed in glial cells or their developmental precursors.

In collaboration with Dr. H-Y Yang, efforts have begun to clone cDNA for precursor(s) to two anti-analgesic neuropeptides isolated by her.

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Project Description

Objectives

The pentapeptides Met-enkephalin and Leu-enkephalin are endogenous opiate-receptor ligands that modulate the release of neurotransmitters in widely distributed neuronal pathways of the central nervous system and gut. We previously cloned and sequenced the cDNA for rat preproenkephalin (ppEnk) and reported the gross distribution of ppEnk mRNA in the rat brain. Our current major objective is to study the regulation of ppEnk gene expression in neuronal systems such as rat brain and clonal cell lines. A particular goal is to identify the control of ppEnk gene transcription by hormones and second messengers such as cAMP and calcium ions. Knowledge about the control of ppEnk gene expression will help in the understanding of endogenous mechanisms of pain perception and neural control.

Dr. H-Y Yang of the Laboratory of Preclinical Pharmacology of the NIMH has recently isolated and sequenced two peptides from bovine brain that react with antibodies against the molluscan cardioexcitatory peptide Phe-Met-Arg-Phe-NH_2. We have begun a collaboration with Dr. Yang to obtain cDNA clones for these peptides in order to characterize the precursor(s) and their mRNA(s). This basic information will help to understand the role of these peptides in pain perception and in normal physiology.

Methods Employed

Messenger RNA purification, RNA Northern blot and dot-blot hybridization analysis, culture of clonal cells, cDNA cloning, colony hybridization with oligonucleotides, DNA sequencing, preparation of RNA (sense or antisense strand) from plasmids containing the SP6 RNA-polymerase promoter.

Major Findings

A. Positive regulation of preproenkephalin gene expression by glucocorticoids in neuroblastoma x glioma hybrid cells.

An established cell line that produces enkephalin and that grows in a chemically defined medium was sought as a simple system to study the regulation of ppEnk gene expression. NG108-15 mouse neuroblastoma x rat glioma hybrid cells contain very low amounts of enkephalin, possess many neuronal properties which are enhanced by prolonged elevation of intracellular cAMP, and grow in a defined serum-free medium. When total RNA from NG108-15 cells was electrophoresed, blotted, and hybridized with ^{32}P-labeled nick-translated rat ppEnk cDNA, a single band of 1500 bases was detected, which is in accord with the size of ppEnk mRNA from brain. To estimate absolute rather than relative abundances of this mRNA, Northern blots were calibrated with known amounts of a synthetic RNA standard containing the coding sequence of ppEnk mRNA. The RNA standard was obtained by transcription of a pSP65-derived plasmid containing a ppEnk cDNA insert. By this method, the abundance of ppEnk mRNA of untreated NG108-15 cells was found to be 20-120 fg/μg total RNA (1.5-10 molecules/cell). In contrast, the ppEnk mRNA abundance of rat striatum, an area of brain rich in enkephalin, was 45 pg/μg RNA.

Treatment of NG108-15 cells with physiological concentrations of glucocorticoids hormones elevated the ppEnk mRNA abundance. For example, treatment with 1 μM dexamethasone (Dex) elevated the mRNA after a 4 hr lag to 3 times the control at 32 h of treatment. Longer treatments slowly elicited greater increases, up to 9 times the control at 8 days. Half-maximal and maximal increases were obtained with 10^{-8} M and 10^{-6} M Dex, respectively. The naturally occurring glucocorticoids hydrocortisone and corticosterone were as effective as Dex at 1 μM, while deoxycorticosterone was weakly effective, and sexual steroids were ineffective at 1 μM. These characteristics are typical of glucocorticoid-receptor mediated responses. The content of Met-enkephalin (Met-enk) immunoreactivity in the cells (20 fmoles/mg protein in untreated cells) was elevated to 2-5 times the control by treatment with Dex for 5 days.

Treatment of NG108-15 cells with 1 mM 8-Br-cAMP or an adenylate cyclase activator such as prostaglandin E_1 (10 μM) or forskolin (10 μM), in the absence of added glucocorticoid, resulted in small elevations of the ppEnk mRNA abundance to twice the control or less in serum-supplemented medium. These compounds elicited no increase in cells adapted to serum-free medium. Forskolin treatment for 5 days also had little or no effect on Met-enk immunoreactivity.

In contrast, treatment of NG108-15 cells with both Dex and either 8-Br-cAMP or adenylate cyclase activation elicited marked elevations of ppEnk mRNA that were greater than the sum of the separate effects: treatment for 24 hr or 8 days elevated the level to 5-8 times or 30 times the level of untreated control cells, respectively. Combined treatment for 5 days also elevated Met-enk immnoreactivity levels to 11-42 times the control. Similar increases were obtained with cells cultured in serum-free medium, indicating that additional hormones are not required for the effect of glucocorticoids + cAMP on ppEnk mRNA. After treatment of cells with Dex + 8-Br-cAMP, withdrawal of these compounds resulted in a loss of 75% of the increase in the ppEnk mRNA abundance over the following 24 hr.

The increases elicited by a 6-hr treatment with Dex, 8-Br-cAMP, and both were almost totally blocked by actinomcyin D, a specific inhibitor of trranscription. Cycloheximide, a specific inhibitor of protein synthesis, unexpectedly enhanced by 70-100% the increases by Dex ± 8-Br-cAMP. These results indicate that (1) the effects of Dex ± 8-Br-cAMP involve transcription of the ppEnk gene, and (2) synthesis of an intermediate protein factor is not required for the effect on the ppEnk gene.

B. Regulation of preproenkephalin gene expression in C6 rat glioma cells.

The C6-BU1 rat glioma cell line, which is one of the parent lines of the NG108-15 hybrid line, was found to contain a relatively high abundance of ppEnk mRNA (3-6 pg/μg RNA or 150-300 molecules/cell), while the other parent, the N18-TG2 mouse neuroblastoma line, had little or no ppEnk mRNA. This result was unexpected since C6-BU1 cells, as well as glial cells in the central nervous system, contain negligible amounts of enkephalin peptides. The C6 line, from which C6-BU1 was derived, also has a high ppEnk mRNA abundnace.

Studies on the regulation of ppEnk gene expression in C6 cells have been initiated using the method of dot-blot hybridization with the rat ppEnk cDNA probe. C6 cells possess beta-adrenergic receptors coupled to adenylate cyclase; thus catecholamines such as (-)-norepinephrine (NE) and (-)-isoproterenol produce a large but transient increase in intracellular cAMP levels. Treatment

of C6 cells with NE (10 μM) alone increased ppEnk mRNA abundance to 3 times the control. Dex (1 μM) alone showed little or no effect, in contrast its effect on NG108-15 cells. Treatment with NE + Dex caused a marked increase of ppEnk mRNA to 7 times the control during 6-10 hr of treatment. The elevations were transient and greatly reduced by 24 hr of treatment. Half-maximal and maximal stimulation by NE in the presence of Dex were obtained with 8.4×10^{-8} M and 10^{-5} M NE, respectively. The stimulation of ppEnk mRNA by NE was blocked by propranolol, a beta-receptor blocker, and not by phentolamine, an alpha-receptor blocker.

Forskolin, which activates adenylate cyclase by interacting with the catalytic subunit, elicited effects similar to NE; the maximal ppEnk mRNA level in the absence and presence of Dex (1 μM) was 2.3 and 5.7 times the control, respectively. The increased ppEnk mRNA elicited by forskolin was transient, as with NE, suggesting that C6 cells have a mechanism for attenuating the response to cAMP elevation.

Actinomycin D completely blocked the increase of ppEnk mRNA caused by NE + Dex, indicating the involvement of transcription, while cycloheximide had no effect. Colchicine, an inhibitor of microtubular assembly, doubled the stimulation by NE + Dex by an unknown mechanism.

In summary, C6 cells contain considerable ppEnk mRNA but little enkephalin, indicating a possible defect in translation of ppEnk mRNA, post-translational processing of proenkephalin, or granular storage of enkephalins. ppEnk gene expression is positively regulated by glucocorticoids and cAMP acting synergistically. The beta-receptor mediated regulation of ppEnk gene expression may represent a physiological process operating in some types of enkephalinergic cells. The high abundance of ppEnk mRNA in a glioma cell line raises the possibility that the enkephalin gene is expressed in normal glial cells or their precursors during development.

C. Biosynthesis of mammalian tachykinins.

As described in last year's annual report, cDNA clones coding for the precursors of the tachykinin neuropeptides substance P and neuromedin K were sought by screening cat dorsal root ganglion and rat spinal cord cDNA libraries with heptadecanucleotides. Several positive clones with similar restriction patterns were obtained that bound the probes under supposedly stringent conditions. Two clones were sequenced and found to contain no open reading frame or region closely matching the probes. It was concluded that the clones obtained were false positives and that it is necessary to screen a library with at least two non-identical oligonucleotide probe pools to reduce the selection of false positives. The project currently is inactive because of lack of manpower.

D. Cloning of cDNA for novel anti-analgesic peptides.

Two peptides that reacted with antisera to the molluscan cardioexcitatory peptide Phe-Met-Arg-Phe-NH_2 were isolated this year from bovine brain by Dr. H-Y Yang of the Lboratory of Preclinical Pharmacology, NIMH. Dr. Yang characterized them as an octapeptide having the sequence Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2 and an octadecapeptide having the sequence Ala-Gly-Glu-Gly-Leu-Ser-Ser-Pro-Phe-Trp-SerLeu-Ala-Ala-Pro-Gln-Arg-Phe-NH_2. Both peptides have anti-analgesic activity when injected into mouse or rat CSF. A cDNA library of 140,000 clones was prepared from bovine hypothalamus mRNA by Dr. Yang and us.

This library is being screened with four sets of tetradecanucleotide probe pools, two for each peptide, synthesized by Dr. Robert Lee, previously of the NIMH and now at the Naval Research Institute.

Significance to Biomedical Research and the Program of the Institute

Enkephalinergic neurons are thought to be important in pain perception, stress, neuroendocrine regulation, central regulation of blood pressure and respiration, and possibly behavior. The mechanisms of regulation of enkephalin biosynthesis at the genetic level are important for understanding the physiological functions of enkephalins. Because of the widespread use of rodents in neurochemical/pharmacological research, the rat ppEnk cDNA probe constructed by us is proving to be a highly useful tool for quantitating the low amount of ppEnk mRNA in rodent brain and rodent cell lines. So far, 15 laboratories around the world have requested and received this probe from us.

The finding of synergistic positive regulation of NG108-15 and C6 ppEnk gene expression by glucocorticoids and hormones or other effectors that elevate cAMP suggest modes of regulation of enkephalin biosynthesis in enkephalinergic neurons. For example, in stress, elevated cortisol and possibly circulating catecholamines may stimulate the biosynthesis and release of enkephalins, which may modulate the stress response. As another example, in neuronal development glucocorticoid levels may affect the timing of the biosynthesis of enkephalin. To generalize beyond the enkephalin system, the results suggest that other neuron-specific genes may be coordinately regulation at the transcriptional level by glucocorticoids and cAMP.

Proposed course

1. Transcription run-off experiments to demonstrate a direct effect of glucocorticoids and cAMP on ppEnk gene transcription.
2. Determination of the regions of the rat ppEnk gene required for glucocorticoid and cAMP regulation by promotor deletion/expression studies.
3. Studies on the effect of calcium ions on ppEnk gene expression in clonal cell lines.
4. Further analysis of the ppEnk system in C6 cells. Search for ppEnk mRNA in other glioma cell lines and human glioma tumors.
5. Continued studies on the regulation by hormones and drugs of ppEnk mRNA concentrations in brain and cell lines.
6. Isolation of clones coding for the Yang anti-analgesic peptides.

Publications

1. Yoshikawa, K., Williams, C., and Sabol, S.L.: Rat brain preproenkephalin mRNA: cDNA cloning, primary structure, and distribution in the central nervous system. J. Biol. Chem. 259: 14301-14308, 1984.

2. Yoshikawa, K., Hong, J.S., and Sabol, S.L.: Electroconvulsive shock increases preproenkephalin mRNA abundance in the rat hypothalamus. Proc. Natl. Acad. Sci. U.S.A. 82: 589-593, 1985.

3. Hong, J.S., Yoshikawa, K., Kanamatsu, T., and Sabol. S.L.: Modulation of striatal enkephalinergic neurons by antipsychotic drugs. Fed. Proc. 44: 2535-2539.

4. Hong, J.S., Yoshikawa, K., Kanamatsu, T., McGinty, J.F., Mitchell, C.L., and Sabol, S.L. Repeated electroconvulsive shocks alter the biosynthesis of enkephalin and concentration of dynorphin in the rat brain. Neuropeptides 5, 557-560, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00151-15 LBG

PERIOD COVERED

October 1, 1984-September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Biology of Cyclic Nucleotides in E. coli

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Alan Peterkofsky, Research Chemist, Macromolecules Section, LBG, NHLBI, NIH
Ellen Liberman, Staff Fellow, LBG, NHLBI, NIH
James Harman, Staff Fellow, LBG, NHLBI
Prasad Reddy, Senior Staff Fellow, LBG, NHLBI, NIH
Jonathan Reizer, Senior Staff Fellow, LBG, NHLBI, NIH

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Macromolecules Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5	4	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In Escherichia coli, adenylate cyclase activity in toluene-treated cells can be inhibited by glucose while the activity in broken cell preparations cannot. Adenylate cyclase activity in the permeabilized but not in broken cells is stimulated somewhat specifically and additively by potassium and phosphate. Kinetic studies show sigmoid substrate-velocity curves for the toluene-treated cells but hyperbolic curves for the broken cells. The stimulatory effects of potassium and phosphate on adenylate cyclase activity in toluene-treated cells are associated with increases in the Vmax and Km for ATP. While the enzyme activity in toluene-treated cells shows a preference for magnesium over manganese, the reverse is observed in broken cells. Stimulation of adenylate cyclase activity in toluene-treated cells requires the presence of the proteins of the phosphoenolpyruvate:sugar phosphotransferase system(PTS). The PTS proteins can be phosphorylated in a P-enolpyruvate-dependent reaction. The stimulatory effects of ions will not occur if the PTS proteins are not phosphorylated. Since potassium phosphate stimulates both adenylate cyclase and PTS activities in toluene-treated cells, it is proposed that the effect of potassium phosphate on adenylate cyclase activity is mediated through an effect on the PTS. A model for dual regulation by glucose of adenylate cyclase activity is proposed. This model involves regulation of both the condition of PTS proteins as well as the cellular concentration of phosphate.

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Project Description:

Objectives:

In Escherichia coli, cAMP plays a crucial role in gene expression. The major mechanism by which cellular cAMP levels are regulated appears to involve a sugar-dependent inhibition of adenylate cyclase activity. While the details remain to be elucidated, it has been established that the sugar-transport pathway called the phosphoenolpyruvate:sugar phosphotransferase system (PTS) is involved in the sugar-dependent inhibition of adenylate cyclase activity.

The regulatory mechanism of adenylate cyclase has been partially defined in studies of permeable cells but has not been reproduced in a cell-free system. In continuing studies of the regulation of adenylate cyclase activity, the effects of anions and cations on the system were examined; it was found that, in addition to the expected requirement for magnesium, the regulated form of the enzyme also requires potassium and phosphate for full activity.

Methods employed:

All studies were done in E. coli strains. The strains were converted to forms that express high levels of adenylate cyclase activity by transformation with a plasmid encoding the gene for adenylate cyclase. Adenylate cyclase and PTS activities were measured in permeable cells and broken cell preparations of wild-type and mutant strains under a variety of conditions.

Major findings:

Permeable cells of a wild-type strain of E. coli had adenylate cyclase activity that was stimulated by potassium ion. The stimulation was specific since it could not be replaced by lithium ion, although it could be partially replaced by rubidium ion. The activity in permeable cells was stimulated by phosphate ion. This stimulation was also specific since it could not be replaced by sulphate ion. The stimulations by potassium and phosphate were independent and additive. In the presence but not in the absence of potassium ion, arsenate could substitute for phosphate. Kinetic studies showed that, in permeable cells, potassium ion and phosphate ion affected both the Km and Vmax for adenylate cyclase. The substrate-velocity curves for adenylate cyclase in permeable cells were sigmoid, consistent with the interpretation that the enzyme is complexed to other factors. The enzyme in permeable cells shows a preference for magnesium over manganese while the enzyme in extracts behaves oppositely. While potassium phosphate stimulates adenylate cyclase activity in permeable cells of wild-type E. coli, this is not observed in similar preparations of a strain in which the genes for the proteins of the transport system are deleted. In one of the unique strains examined, potassium phosphate stimulated both adenylate cyclase and PTS activities. These findings were interpreted in the framework of a functional complex of adenylate cyclase with the proteins of the sugar-transport system.

Significance to biomedical research and the program of the Institute:

Growth regulation is a central mechanism in biology. Adenylate cyclase is a most important enzyme in this process. It is therefore of great interest to develop a detailed understanding of the mechanism of this enzyme.

Proposed course:

It is our intention to continue to study the mechanism of the regulation of the activity of adenylate cyclase in E. coli. It appears that the cellular form of the enzyme is as a complex with both some physiologically relevant ions and proteins of the sugar transport system. Our efforts will be concentrated on the purification of adenylate cyclase and the transport proteins with a view to in vitro reconstitution of the functional multienzyme complex.

Publications:

1. Liberman, E., Reddy, P., Gazdar, C. and Peterkofsky, A.: The Escherichia coli Adenylate Cyclase Complex: Stimulation by Potassium and Phosphate. J. Biol. Chem. 260: 4075-4081, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00152-11 LBG

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Metabolism of Peptide Hormones

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Alan Peterkofsky, Research Chemist, Chief, Macromolecules Section, LBG, NHLBI, NIH
Fiorenzo Battaini, Visiting Associate, LBG, NHLBI, NIH

COOPERATING UNITS *(if any)*

Priscilla Dannies, Dept. of Pharmacology, Yale Univ.

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Macromolecules Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cyclo(Histidyl-Proline) is a metabolite of thyrotropin-releasing hormone. It has been suggested by other workers that this peptide plays a role in regulating prolactin secretion in GH cells. The current investigation of the effect of cyclo(His-Pro) on GH cells indicated that it does not affect basal prolactin release or accumulation or the levels stimulated by TRH. cAMP levels in GH cells are elevated by TRH or VIP, but not influenced by cyclo(His-Pro). cGMP levels in GH cells are not affected by either TRH or cyclo(His-Pro). While there is specific binding of TRH to receptors in GH cells, no such receptors for cyclo(His-Pro) are detectable. It is suggested that GH cells are unresponsive to cyclo(His-Pro).

Project Description:

Objectives:

Thyrotropin-releasing hormone (TRH) is a hypothalamic releasing factor which has been shown to stimulate the release of prolactin. Histidyl-Proline diketopiperazine (cyclo(His-Pro) is formed by metabolism of TRH in the brain and blood and produces a variety of biological effects both centrally and in the pituitary gland. Of major interest to studies dealing with the mechanism controlling prolactin secretion were the reports that cyclo(His-Pro) inhibits prolactin release under several conditions in vivo in rats and monkeys and in vitro in human pituitary tumor cells; conflicting results were obtained by others. GH cells are cloned rat pituitary tumor cells that can be stably maintained in tissue culture. Prolactin release in GH cells was reported to be inhibited by cyclo(His-Pro). It was the purpose of the present studies to validate and further explore these findings.

Methods employed:

GH cells were grown in culture. Prolactin concentrations were measured by an immunological procedure. The cyclic nucleotides cAMP and cGMP were measured by a binding protein assay and a radioimmunoassay, respectively. Putative membrane receptors were determined by the binding of tritium-labeled TRH or cyclo(His-Pro) to intact cells or homogenates of the GH cells.

Major findings:

Exposure of GH cells to TRH resulted in an increase in the release and accumulation of prolactin. Cyclo(His-Pro) had no effect on the release or accumulation of prolactin in either the absence of presence of TRH. TRH increased the levels of cAMP in GH cells. Cyclo(His-Pro) had no effects on cAMP levels in either the absence or presence of TRH. A similar unresponsiveness of GH cells to cyclo(His-Pro) was observed with VIP, which greatly increased cAMP levels in the absence or presence of cyclo(His-Pro). cGMP levels in GH cells were not affected by TRH or cyclo(His-Pro) alone or in combination. While binding of tritium-labelled TRH to both cells and homogenates of GH cells could be demonstrated, no such binding of labelled cyclo(His-Pro) was found.

Significance to biomedical research and the program of the institute:

Cyclo(His-Pro) has been shown to have a variety of biological activities. An understanding of the mechanism by which these activities are accomplished will help to understand human physiology better.

Proposed course:

Project concluded.

Publications:

1. Battaini, F., Dannies, P.S. and Peterkofsky, A.: Unresponsiveness of GH Cells to Cyclo(Histidyl-Proline), a Metabolite of Thyrotropin Releasing Hormone. Life Sciences 35:2519-2527, 1984.

Annual Report
Section on Enzymes
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1985

Role of Oxygen Radical Mediated Inactivation of Enzymes in Protein Turnover, Neutrophil Function, and Gliosis

(a) Protein Turnover. In previous studies, it was shown that a number of different biological and nonenzymatic mixed-function oxidation systems catalyze the covalent modification of enzymes and thus render them susceptible to proteolytic degradation by diverse proteases. Alkaline proteases which degrade the oxidized but not the native form of glutamine synthetase have been purified to homogeneity from mouse and rat liver cytosol and from E. coli extracts. The nonlysosomal liver enzyme has a molecular weight of 650,000 and is composed of multiple subunits ranging in size from 22,000 to 34,000 daltons. This protease is inhibited by thiol reagents (phenylmethylsulfonyl fluoride) and partially by aprotenin, leupeptin, antipain and chymostatin, but not by α-antitrypsin, soybean trypsin inhibitors, or inhibitors of metalloproteins. Based on its endopeptidase activity toward the insulin B chain and its susceptibility to various inhibitors, this alkaline protease is classified as a cysteine protease. The rat liver protease also selectively degrades the oxidized forms of rabbit muscle enolase and pyruvate kinase, and preferentially degrades the oxidized forms of phosphoglycerate kinase and creatine kinase.

The protease from E. coli is a monomeric protein (M_r = 75,000). It cleaves a 4,000 M_r peptide from the carboxylterminus of oxidized glutamine synthetase. It also degrades less readily the acetylated and carboxymethylated derivatives of glutamine synthetase, the B chain of insulin, but not hemoglobin. Its activity is inhibited by metal chelators and partially by aprotinin but not by the other inhibitors mentioned above. The rat liver and bacterial proteases represent a new class of proteases most likely concerned with nonlysosomal mechanisms of protein turnover.

Studies of the time-dependent changes that occur during exposure of glutamine synthetase to a MFO system comprised of O_2, Fe(III) and ascorbate revealed: (i) that the loss of enzyme activity is associated with the loss of a single histidine residue per subunit; (ii) the generation of carbonyl groups is significantly slower and reaches a maximum of one equivalent per subunit; (iii) the susceptibility to degradation by the nonlysosomal protease of rat liver lags behind both (i) and (ii) and is correlated with the loss of a second histidine residue per subunit.

(b) Mechanism of MFO-catalyzed Enzyme Modification. Cyanogen bromide treatment of glutamine synthetase yields a pentapeptide which in the native enzyme has the sequence -Met-His-Cys-His-Met-. From a comparison of the pentapeptide isolated from native and oxidized forms of glutamine synthetase, it is deduced that the histidine residue on the N-terminal side of this peptide is converted to an asparagine residue duirng exposure to MFO systems.

A highly sensitive method for measuring the carbonyl content of proteins has been developed by taking advantage of the fact that treatment with sodium

borotritide leads to reduction of the carbonyl groups to their corresponding hydroxyl derivatives and to the incorportation of one equivalent of non-exchangeable tritium per carbonyl group initially present. Moreover, following acid hydrolysis of the tritiated protein, the constituent amino acids can be separated by chromatography, and those derived from carbonyl derivatives can be readily detected on the basis of their radioactivity. With this technique, it could be ascertained that at least four different amino acid residues in either glucose-6-phosphate dehydrogenase or glutamine synthetase are converted to carbonyl derivatives by exposure to an MFO system. Based on similarities in the chromatographic behavior of radioactive amino acids obtained when poly-L-histidine and poly-L-lysine are exposed to identical treatments, it is tentatively concluded that lysine and histidine residues in proteins are among those readily oxidized by MFO systems.

(c) Oxidative Deamination of Amino Acids. An MFO system comprised of chelated Fe(II) or Fe(III) and H_2O_2 (the Fenton reagent) was found to catalyze the oxidation of the common amino acids to their corresponding α-keto acid derivatives and ammonia. From a detailed study of the oxidation of leucine, it was established that: (i) oxidative deamination requires the presence of both chelated and nonchelated iron; (ii) oxidative deamination is almost completely dependent upon the presence of bicarbonate ion; (iii) any one of a large number of chelating agents including citrate and nucleoside di- and tri- phosphates will stimulate oxidative deamination so long as they are not present in excess; (iv) O_2 is a by-product of the H_2O_2-mediated oxidation. These results are consistent with mechanisms in which hydroxyl radical, superoxide anion, and possibly bicarbonate radicals are intermediates.

(d) Neutrophil Function. When human neutrophils are exposed to any one of a variety of agents including bacterial chemotatic peptide (fMet-Leu-Phe, fMLP), latex beads, phorbol myristate acetate (PMA), etc., they undergo a period or oxidative burst. This reflects a shift in metabolism from glycolysis to the hexose monophosphate pathway and the activation of an NADPH oxidase, and results in the generation of a number of activated oxygen species, i.e., O_2^-, $^{\cdot}OH$ and H_2O_2. The possibility that oxidative inactivation of enzymes contributes to the killing of bacteria by neutrophils is supported by previous studies showing that during the period of oxidative burst, neutrophils catalyze the inactivation of exogeneously supplied pure glutamine synthetase as well as the glutamine synthetase present within E. coli cells. In addition, the possibility that enzyme oxidation is implicated in the ultimate destruction of the nuetrophils themselves is supported by the fact that during oxidative burst some amino acid residues of endogenous neutrophil proteins are converted to carbonyl derivatives and several endogenous enzymes are inactivated. It has now been found that when neutrophils from normal individuals are activated by PMA, but not by fMLP, in the presence of ^{14}C-labeled tyrosine, the tyrosine becomes covalently bound to endogenous protein. The likelihood that protein tyrosylation is mediated by the NADPH oxidase activity as in activated neutrophils from individuals with chronic granulomatous disease fail to exhibit this phenomenon, but neutrophils from individuals with hereditary myeloperoxidase deficiency do. This suggests that the protein-tyrosine interaction is mediated by NADH-oxidase activity and is independent of protein synthesis. Further studies show that tyrosine potentiates the generation of protein carbonyl groups during the period of oxidative burst and that attachment of tyrosine to proteins does not involve extensive cross-linking of peptide chains as is elicited by an H_2O_2-peroxidase-dependent mechanism in the early stages of egg fertilization.

(e) Protein Kinase C Activation. The possibility that activation of neutrophils involves phosphorylation of intracellular proteins is suggested by the fact that PMA and other phorbol esters which activate neutrophils also activate the calcium-dependent protein kinase C.

In an effort to identify C kinase-dependent phosphorylated substrates, ^{32}P protein-labeling patterns were examined by SDS gel electrophoresis of neutrophil extracts prepared from PMA activated cells. Approximately 8 protein bands ranging from 15 kd-140 kd were phosphorylated with $^{32}P\gamma$-ATP in the presence of phospholipid and Ca^{++} and 2 bands (40 kd and 15 kd) were especially prominent. Similar studies have been carried out with HL-60 cells. These cells can be differentiated by a variety of agents which give rise to different end stage type cells. DMSO and retinoic acid promote a myelocytic type of differentiation with the formation of a neutrophil-like cell. When cells are treated in culture with 1% DMSO for 4 to 6 days, respiratory burst activity can be detected as early as 24 hours. Moreover, during differentiation with DMSO, HL-60 cells exhibit a progressive increase in protein kinase C-dependent phosphorylation. Under these conditions, again about 8 protein bands are phosphorylated and again 2 bands (40 kd and 15 kd) were prominent. It is noteworthy that the differentiation of HL-60 cells induced by retinoic acid leads to little or no phospholipid and Ca^{++}-dependent phosphorylation, and to little or no respiratory burst competence as determined by cytochrome C reduction.

In contrast, when HL-60 cells are treated with PMA, they differentiate into macrophage-like cells which exhibit respiratory burst activity, but of longer duration and lower intensity than that of neutrophils. Under these conditions, a different phosphorylation pattern is observed, but again the 40 kd band is prominent.

(d) Gliosis. Dr. Halks-Miller of Stanford University has shown that under specified conditions dispersed brain cells undergo reorganization in vitro forming spheres containing a central core of neuronal elements and an outer layer of glial cells. Subsequent injury of the neuronal cells triggers growth and migration of the glial cells to the site of injury. Histological examination of the injured spheres reveals extensive accumulation of glial cells with concomitant damage to to the neuronal cells. This process resembles the pathology of gliosis and glial scarring in vivo. Moreover, this process is accompanied by the production of activated oxygen species and the generation of malondialdehyde (presumably from lipid peroxidation). In preliminary experiments carried out in collaboration with Dr. Halks-Miller, it has been demonstrated that gliosis in this model system is associated with the generation of very high levels of protein carbonyl groups.

Annual Report
Section on Intermediary Metabolism
and Bioenergetics
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1985

A. A continuing research project deals with the role(s) of selenocysteine in selenium-dependent enzymes and the mechanism of incorporation of this unusual amino acid in proteins. A selenium-dependent clostridial glycine reductase utilizes glycine as terminal electron acceptor and synthesizes ATP concomitant with the reduction of glycine to acetate and ammonia. The soluble enzyme complex consists of three proteins: a 12,000 dalton selenoprotein (protein A) that contains one selenocysteine residue, a 200,000 dalton carbonyl group protein (protein B), and a slightly larger, very hydrophobic protein (protein C). Methods for isolation of proteins A and B in pure form were developed previously. Finally, using hydrophobic chromatographic matrices, highly purified, stable preparations of protein C have been obtained. This colorless protein is not sensitive to radical scavengers and does not appear to contain iron. Since all isolation procedures for the three protein components can be scaled up readily, it now should be feasible to use the reconstituted enzyme complex as a reagent for studies on mechanism of ATP synthesis and role of the selenocysteine residue in the overall reaction.

The biological mechanism of formation of selenocysteine residues in selenium-dependent enzymes is investigated in two different bacterial systems. One approach is to determine the nucleotide sequence of the DNA that encodes the selenocysteine-containing polypeptide. This should identify the precursor amino acid that is presumed to be modified post-translationally to form selenocysteine. The selenoprotein A of glycine reductase has been partially sequenced around the single selenocysteine residue by automated Edman degradation. The sequence of the tryptic peptide is [-Val-Secys-Thr-Ala-Ala-Gly-Ala-Met-Asp-Leu-Glu-Asn-Gln-Lys-]. In this case a synthetic RNA probe that matches a part of this sequence could then be used to isolate the complimentary DNA fragment. Unfortunately, the high redundancy in the codes of the amino acids flanking the selenocysteine residue will require a large number of synthetic oligonucleotides unless educated guesses can eliminate some of the possibilities. High titre antibodies to selenoprotein A were raised by using protein A covalently coupled to serum albumin as antigen. Using sensitive dot-immunoassays and immunoblotting of SDS-polyacrylamide gels, no precursor forms of the protein A were detected in cells grown under selenium deficient conditions whereas ^{75}Se-labeled selenoprotein A could be detected at extremely low levels by the same assay procedure.

Escherichia coli, when grown anaerobically, produce two types of formate dehydrogenases that contain selenocysteine. One of these, produced when nitrate is electron acceptor, forms a complex with nitrate reductase and the other, produced in the absence of nitrate, is part of a formate-hydrogen lyase complex. The selenoprotein subunits of these two formate dehydrogenases are distinct gene products. The DNA encoding the 80,000 dalton selenopeptide of the formate-hydrogen lyase complex has been cloned into a plasmid bearing a chloramphenicol resistance marker by Dr. A. Böck of the University of München. A DNA fragment that encodes a 60,000 dalton truncated selenocysteine peptide also has been cloned and is being sequenced. In a collaborative effort with Böck we have produced and isolated the ^{75}Se-selenoprotein subunit (80,000 daltons) produced by his plasmid bearing E. coli strain. From this we will prepare and isolate ^{75}Se-labeled peptides for amino acid sequence determination. Comparison of the corresponding DNA and amino acid sequences flanking the selenocysteine residue should provide information as to its origin.

B. The specific occurrence of selenium in amino acid transfer ribonucleic acids (Se-tRNAs) in several anaerobic bacteria and E. coli was demonstrated in previous studies from this laboratory. The major selenium-containing nucleotide in several of these tRNAs was identified as 5-methylaminomethyl-2-selenouridine [mnm^5-Se^{2}U]. This nucleotide, which is located in the wobble position of the anticodon of a tRNA^{Glu} from Clostridium sticklandii, is essential for enzymic esterification of this tRNA with its cognate amino acid. The seleno-tRNA^{Glu} interacts similarly with the two glutamate codons, GAG and GAA, whereas the corresponding thio-tRNA^{Glu} from E. coli shows a high preference for GAA. When these tRNA species, charged with radioactive glutamate, were compared in an in vitro protein synthesis system (wheat germ + rabbit globin mRNA), glutamate esterified to the Se-tRNA^{Glu} was incorporated into protein with a 40% greater efficiency than when esterified to S-tRNA^{Glu}. Since rabbit globin mRNA, like many eukaryotic mRNAs, has a greater abundance of glutamate codons ending in G rather than A, these results indicate that more efficient translation of other cloned eukaryotic messages would be observed if Se-tRNAs are used. In E. coli, Se-tRNA^{Glu} and Se-tRNA^{Lys} are produced if culture media contain 0.1 to 1 μM levels of selenium whereas in unsupplemented minimal media only the corresponding thiol tRNAs, which show a low preference of G-ending codons, are present. Preliminary studies on the biosynthesis of 2-selenouridines indicate that selenium may replace sulfur already introduced in the 2-position. A requirement for ATP suggests that sulfur is activated to become a leaving group prior to its replacement with selenium. A family of 2-selenouridines containing various types of substituents at the 5-position of the uridine ring have been detected in the tRNAs of certain E. coli mutants and various anaerobic bacteria. One of these was shown to be indistinguishable from 5-aminomethyl-2-selenouridine which was synthesized as a reference compound. Improved methods have been developed for synthesis of related nucleosides in this series to serve as reference compounds.

C. Methanococcus vannielii, in common with other methane-producing bacteria, contains conspicuous amounts of 8-hydroxy-5-deazaflavin, a coenzyme that resembles flavins in structure. Previous studies from this laboratory have characterized three enzymes that utilize 8-hydroxy-5-deazaflavin as cofactor and the stereochemistry of these hydrogen transport processes has been elucidated. To determine the mechanism of biosynthesis of the deazaflavin, labeling experiments have been carried out using [^{14}C]guanine derivatives. In crude enzyme preparations both guanine and GTP served as efficient precursors and in both cases the 8-carbon of the guanine moiety is eliminated as carbon dioxide. Using release of [^{14}C]carbon-8 as an assay, an oxygen sensitive enzyme that catalyzes an early step in the biosynthetic series of reactions has been partially purified. The possible role of this enzyme in the biosynthesis of methanopterin, a pterin also present in high amounts in methane bacteria, is of interest. Since the N at position 5 of the isoalloxazine ring of flavins is replaced by a methylene group in the deazaflavins, the point of divergence of the biosynthetic pathways leading to these two types of coenzymes is not readily predictable. However, the efficient use of GTP with loss of carbon-8 of the guanine moiety for synthesis of the deazaflavin indicates that early steps of the two pathways are similar.

D. In a research project on the conversion of biomass to methane supported by the Gas Research Institute of Chicago, we are studying at the enzyme level the conversion of acetate and one-carbon compounds to methane. In the biosynthetic direction it has been shown that carbon monoxide can serve as precursor of the carboxyl group of acetate and this is mediated by an enzyme that exhibits carbon monoxide dehydrogenase activity. Indirect implication of a similar enzyme in acetate conversion to methane and carbon dioxide prompted us to undertake isolation and a detailed study of a carbon monoxide dehydrogenase from Methanosarcina barkeri. Characterization of this oxygen-sensitive, nickel enzyme is in progress.

E. In a project undertaken to detect and characterize anaerobic radical-mediated enzymic reactions, it was found that a diol dehydratase from Clostridium glycolicum differs from previously studied B_{12} coenzyme-dependent diol dehydratases. The C. glycolicum enzyme preparations exhibit a radical signal which correlates with diol dehydrastase activity, but in this case no B_{12} coenzyme appears to be involved. The new diol dehydratase is extremely oxygen labile and is particulate. All commonly used procedures for solubilization of membrane bound enzymes failed to release the diol dehydratase. Finally, a soluble preparation was obtained by sonication of the particles at alkaline pH under strictly anaerobic conditions in a hydrophobic zwitterion buffer containing lysolecithin. In addition to its oxygen sensitivity, the enzyme is inhibited by the known radical scavengers, hydroxylamine and hydroxyurea, metal chelating reagents, and thiol reagents. As shown by EPR studies, addition of radical scavengers or thiol reagents to enzyme preparations also dissipated the radical signal.

Annual Report
Section on Metabolic Regulation
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1985

The research activities of the investigators in the Section on Metabiolic Regulation are mainly concerned with the physical and chemical approaches to resolve the mechanisms of enzyme action and its regulation, and to study the antibody-antigen interaction. Currently, the research is concentrated on (1) regulation of enzymic activity by cyclic cascade systems, Ca(II) and Ca(II)-calmodulin complex; (2) overproducing enzymes which are involved in glutamine synthetase cascade by cloning methods; (3) mechanistic studies of enzyme action and activation; and (4) protein-DNA interaction. Together, these research programs will provide a better understanding of how biochemical processes work in cells.

I. Regulation of Enzymic Activity

A. Regulation of the Mg(II)-ATP-dependent Protein Phosphatase

The Mg(II)-ATP-dependent protein phosphatase, inactive as isolated, is a major phosphorylase phosphatase in skeletal muscle and has been found in numerous other tissues. An improved purification procedure has been developed in which TSK-DEAE HPLC column chromatography and TSK-phenyl HPLC column chromatography steps are used. The TSK-DEAE HPLC column step separates a 62 KD_a polypeptide which had previously copurified with enzyme activity and with the 38 and 31 KD_a subunits. However, this chromatographic step failed to remove a 68-70 KD_a protein which can be removed by the TSK-phenyl HPLC chromatographic step. The highly purified enzyme consists of 38 and 31 KD_a subunits. The specific activity of this enzyme is protein concentration dependent. In dilute solution (~ 0.1 mM), the specific activity is 13,000 nmole/min/mg. This value is decreased to about 3,500 nmole/min/mg at high protein concentration (~ 6 nM). These data suggest an association/diassociation mechanism which affects the activity of the activated enzyme.

The activation mechanism involves a transient phosphorylation of the 31 KD_a subunit (also known as modulator or inhibitor-2) catalyzed by kinase F_A (also known as glycogen synthase kinase-3). Phosphorylation of the modulator causes a conformational change in the 38 KD_a catalytic subunit, and leads to the activation of the phosphatase. The active phosphatase is capable of dephosphorylating the phosphorylated modulator causing the phosphatase to return to its inactive form via a slow isomerization step. The time course of the phosphatase activation and of phosphate incorporation into the modulator subunit was investigated. The phosphorylation is rapid, and as phosphatase activation proceeds, there is a decrease in the amount of phosphate incorporation before it reaches a steady state level. A couple lines of evidence in support of the phosphorylation induces a conformational change of the catalytic subunits such that it converts from its inactive to its active form are: (i) removal of the modulator subunit by limiting proteolysis fails to activate the native (inactive) phosphatase unless it has been first phosphorylated; (ii) the modulator can be thiophosphorylated, however, it fails to activate the enzyme. Upon removal of modulator by limiting proteolysis, the enzyme becomes activated. The slow isomerization

step which leads the active enzyme to its inactive form was demonstrated by blocking the activation reaction with EDTA and then following the time course of the inactivation reaction. This reaction is first-order with a $t_{1/2}$ equals to 13-15 minutes.

The inhibition of the Mg(II)-ATP-dependent protein phosphatase by the regulatory subunits of type II cAMP-dependent protein kinase was further investigated. Analysis of the inhibition data, with the assumption that the regulatory subunit binds on a single site on the phosphatase yields apparent K_D of about 100 and 42 nM for reaction mixture containing 400 ng/ml and 80 ng/ml of F_A, respectively. This suggests an antagonism between the binding of regulatory subunit and of kinase F_A to the phosphatase. If one assumed a mutually exclusive mechanism for these bindings, one can estimate a K_D value of ~ 3 nM for F_A binding to phosphatase and K_D for regulatory subunit•phosphatase complex is ~ 24 nM which is quite similar to the K_i (15 nM) for regulatory subunit inhibition of the catalytic fragment of the phosphatase. This seems to support the hypothesis that the regulatory subunit binds to the phosphatase instead of the kiase F_A. In addition, computer simulation of a cyclic cascade system using the cAMP-dependent protein kinase and the Mg(II)-ATP-dependent protein phosphatase as two converter enzymes reveals that they constitute a highly coordinated regulatory cascade. This synchronous regulatory cascade provides additional signal amplification and it enhances the sensitivity in changes of fractional phosphorylation of the interconvertible enzyme in response to increasing cAMP concentration. In addition, the coincident activation of protein kinase and inhibition of protein phosphatase reduces the ATP consumption required for maintaining the function of a cyclic cascade, thus making it more energetically efficient.

B. Glutamine Synthetase Cascades

1. Regulation of the In vivo Synthesis of Glutamine Synthetase by P_{II}. Glutamine synthetase (GS) in E. coli and other enteric bacteria is rigorously regulated in response to the availability of the nitorgen source. The enzymic activity is modulated by (i) feedback control, (ii) covalent interconversion of enzymes, and (iii) repression and derepression of the synthesis of the enzyme. Covalent modification involves two nucleotidylation cycles, namely, the adenylylation and deadenylylation of GS, and the uridylylation and deuridylylation of a regulatory protein, P_{II}. Adenylylation and deadenylylation of GS, which leads to its inactivation and reactivation, respectively, is catalyzed at two separate catalytic sites of adenylyltransferase (ATase). The activity of this bifunctional enzyme is modulated by P_{II}, which undergoes reversible uridylylation/deuridylylation. The unmodified P_{II} stimulates the adenylylation activity of ATase, while the uridylylated P_{II} is required for the deadenylylation reaction. The enzyme which catalyzes the uridylylation and deuridylylation of P_{II} is also a bifunctional enzyme, the uridylyltransferase (UTase). The relative concentration of the four proteins involved in this bicyclic cascade was determined using sheep antibodies prepared against all four antigens and E. coli K-12 grown on a nitrogen-limiting medium containing ^{35}S-methionine. Each protein was precipitated from the crude extracts using the antibodies and further purified on a SDS-polyacrylamide gel. The ^{35}S-methionine was quantitated and then normalized to the amount of methionine in each protein. The results show that the molar ratio of UTase to ATase to P_{II} (tetramer) to GS (dodecamer) is 1:26:11:34.

In addition, it was found that an elevated level of either ATase or UTase due to the presence of multicopy plasmid can affect the in vivo synthesis of GS.

The level of GS in the strain harboring pglnD (plasmid containing structural gene of UTase) or pglnE (plasmid containing structural gene of ATase) is higher than that of the wild type strain by 4- and 3-fold, respectively, when cells were grown on a nitrogen excess minimal medium (4 mM glutamine, 20 mM ammonium chloride, and 0.5% glucose). This observation is consistent with the reported suggestion that the repression of GS synthesis in E. coli required the presence of unmodified P_{II} protein. At high concentrations of UTase and ATase, for which P_{II} is a substrate and a modulator, respectively, the free P_{II} concentration would be low. Therefore the synthesis of GS is derepressed. This hypothesis is in agreement with the observation that in the absence of P_{II} (due to mutation in the structural gene of P_{II}, glnB) GS level is independent of the concentration of UTase and ATase.

2. Purification of Adenylyltransferase and Uridylyltransferase. A simple procedure involving an affinity chromatography step was devised to purify the adenylyltransferase and the uridylyltransferase from E. coli strains which have been engineered to overproduce them. In this respect, the structural gene of adenylyltransferase, glnE gene, and the structural gene of uridylyltransferase, glnD gene, were cloned into a plasmid vector carrying the strong, regulatable λ phage promoter such that their synthesis could be enhanced by several hundred-fold. Homogeneous preparations of these two enzymes were obtained using a simplified procedure which included a DEAE column chromatography step followed by a Matrix gel (Amicon) dye affinity chromatography step. In the latter chromatography, red gel was used for ATase purification, while green gel was used for UTase. The purified UTase, albeit a single band when analyzed on SDS-polyacrylamide gel, gives two protein peaks, one corresponds to the monomeric form and the other represents the oligomeric form which has a molecular weight larger than the hexamer.

3. Mechanistic Studies of the Uridylylation/Deuridylylation Catalyzed by Uridylyltransferase. Steady state kinetic studies show that the monmomeric form and the oligomeric form of the purified uridylyltransferase exhibit similar kinetic properties for the uridylylation reaction, while they are distinctly different for the deuridylylation reaction. In the uridylylation reaction, the two substrates, UTP and P_{II}, bind to the enzyme via a order-mechanism, namely, the binding of UTP is prerequisite for the binding of P_{II}. ATP is an essential activator while α-Kg activates the reaction by increasing both the V_{max} (21-fold) and the affinity of ATP (9-fold). Glutamine is a noncompetitive inhibitor with respect to P_{II}. The requirement of ATP as an activator is very strict, its analogs such as GTP, CTP, ADP, N_3-ATP, Aza-ε-ATP and AMPPNP fail to activate. In the deuridylylation reaction, $P_{II}(UMP)_4$ is the only substrate, glutamine is an activator, it increases the V_{max} and the affinity of $P_{II}(UMP)_4$ by 7- and 4-fold, respectively. CMP is a potent inhibitor, it competes with the binding of $P_{II}(UMP)_4$.

C. Phosphorylation/Dephosphorylation of Ca(II)-Calmodulin-dependent Protein Phosphatase by Protein Kinase C

The calmodulin-dependent protein phosphatase is found to be phosphorylated effectively by Ca(II)-dependent protein kinase C. The phosphorylation is dependent on the presence of Ca(II), phospholipid, and is stimulated by phorbol esters. The stoichiometry is approximately one mole of phosphate incorporated per mole of enzyme. In addition, it is found that the phosphorylated form of calmodulin-dependent protein kinase is a good substrate for the Ca(II)-calmodulin-dependent

protein phosphatase. These findings further point to the intriguing interplay involving Ca(II) regulation and phosphorylation/dephosphorylation of proteins.

II. Mechanism of Enzyme Action and Activation

A. Mechanism of Activation of Calmodulin-dependent Protein Phosphatase by Ni(II) and Mn(II).

The calmodulin-dependent protein phosphatase requires a divalent metal ion such as Ni(II), Mn(II) or Mg(II) to express its catalytic activity. Ca(II) alone, without calmodulin, fails to support the phosphatase activity. However, in the presence of Ni(II) or Mn(II), the enzyme is active without calmodulin, although Ca(II)-calmodulin further stimulates its activity. Ni(II) activation is irreversible and its time course is characterized by an initial lag period, signifying a slow conformational rearrangement. This lag time decreases as Ni(II) concentration is increased and is not affected by varying levels of the substrate, p-nitrophenylphosphate. In the presence of Ca(II)•calmodulin complex, only one Ni(II) is bound and the lag phase conforms with first-order kinetics. Kinetic data suggest that Ni(II) initially forms a loose complex with Ca(II)•calmodulin•phosphatase complex with a dissociation constant of 2.5 mM for Ni(II) and this initial Ni(II)•enzyme•calmodulin complex is inactive. The rate constant for converting this inactive form to its active form is 0.083 sec^{-1}. In the absence of calmodulin, the lag phase for activating the phosphatase by Ni(II) is not a first-order process, it follows a multi exponential function. The Ni(II) binding study shows at least two Ni(II) are bound to each mole of enzyme. Thus, it appears that calmodulin binding prevents the phosphatase from binding a second Ni(II). Activation by Mn(II) in the presence of calmodulin is similar to that by Ni(II) except that the process is reversible. The initial enzyme•Mn(II) binding is tighter, with a K_D of 200 μM, but the activation rate constant, 0.042 sec^{-1}, is slower compared with corresponding parameters for Ni(II).

B. Mechanistic Study of Rabbit Skeletal Muscle Actomyosin ATPase Cycle

Muscle contraction is driven by a cyclic reaction between actin and myosin, coupled to the hydrolysis of ATP. Two models have been proposed to account for the kinetic data of ATP hydrolysis. A four-state model which has four states containing bound ATP or ADP•P_i and the rate-limiting step is ATP hydrolysis. In the six-state model, there are six myosin bound reaction intermediates containing phosphate in the form of ATP or ADP-P_i, and the rate-limiting step is a conformational change which occurs before P_i release but after ATP hydrolysis. A difference between these two models is that only the four-state model predicts that almost no acto-subfragment 1•ADP•P_i (acto-S-1•ADP•P_i) complex will be formed when ATP is mixed with acto•S-1. Results from investigating the amount of acto•S-1•ADP•P_i formed when ATP is mixed with S-1 cross-linked to actin suggests that at both high and low ionic strength the ATP hydrolysis step is not the rate-limiting step. Instead, the data are consistent with the rate-limiting step occurring before P_i release and after ATP hydrolysis step, as proposed in the six-state kinetic model.

III. Model Analysis

A. Theoretical Treatment of Interfacial Reaction Dynamics

Bimolecular reactions in which one of the reactants is localized at an interface while the other reactant is initially molecularly dispersed in the

homogenous phase can occur by two paths. One involves the direct interaction of a homogenous reactant with its interfacially localized reaction partner, and the other proceeds by initial adsorption of the homogenous reactant and subsequent surface diffusion to reaction. A branching method for the treatment of the surface dynamics was developed whereby the total reaction velocity is given by the rate at which reactants enter into a specified, arbitrary configuration, multiplied by the product of the probabilities of the subsequent individual event necessary for reaction to occur. This treatment is particularly useful for the analysis of reactions which occur by multiple mechanisms. Theoretical analysis shows that although rate enhancement as compared to an analogous homogenous reaction (with three-dimensional diffusion) can be anticipated only for very low interfacial concentrations of localized reactant, the two-dimensional surface diffusion mechanisms accounts for an appreciable portion of the total reactivity within a wide range of circumstances for ligand-cell surface receptor interactions. In addition, this study revealed that in systems with many reactive sites localized on a single moiety, e.g., a single polypeptide, the diffusion controlled binding kinetics of ligand to those sites will be nonlinear, thus, apparent kinetic cooperative effects can be observed, even in the absence of site-site interactions.

B. Validity of Rapid Equilibrium Treatment for the Ca(II) Activation of Calmodulin-regulated Enzymes.

The activation of cyclic nucleotide phosphodiesterase by Ca(II) and calmodulin has been treated theoretically with the assumption that all the complexes involved are in rapid equilibria. The on-rate constant for the binding of phosphodiesterase to the Ca(II)•calmodulin complex is $\sim 4 \times 10^6$ $M^{-1}sec^{-1}$ which is reasonably fast. However, the off-rate constant for the Ca(II)•calmodulin complex from enzyme•Ca(II)•calmodulin complex is 3.2×10^{-3} sec^{-1} which is very slow. Therefore, it appears that rapid equilibrium treatment may not be valid for this activation mechanism or similar calmodulin-regulated systems. A mathematical derivation based on a more stringent steady state assumption for the binding of enzyme to various Ca(II)•calmodulin complexes demonstrates that the rapid-equilibrium assumption is valid as long as the on-off rates for the binding of Ca(II) to calmodulin and to various enzyme•calmodulin complexes are fast.

C. Electric Field Induced Energy Transduction and Electroconformational Coupling.

Transmembrane potential plays a large role in determining the activity of membrane bound proteins. The rationale behind the importance of the electric field for such systems lies in the fact that a modest physiological transmembrane potential of 100 mV across a 50 Å membrane represents an electric field strength of 200,000 V/cm, which is a very strong field.

Motivated by the experimental observation that an oscillating electric field induces active transport of Rb^+ ion into erythrocytes via the Na^+-K^+ ATPase, we developed a kinetic model to describe transduction of electric energy. This model, as confirmed by computer simulation and analytical solution, is competent for energy transduction under an externally modulated field. It was also shown that by appropriately timed opening and closing of an ion channel near an energy transducing enzyme system, a metabolically generated DC field can be locally modulated and converted to electrochemical potential energy or chemical bond energy such as synthesis of ATP. This concept can explain experimental results

on the electric field stimulated ATP synthesis by the submitochondrial particle F_oF_1-ATPase. It should be pointed out in this model that the energy coupling event is treated as a field-induced protein conformational change, thereby allowing for the understanding of energy transduction in terms of classical molecular mechanisms of enzyme action.

D. Cyclic Cascade vs Simple Allosteric Control

Metabolic regulation by a cyclic cascade model utilizes both covalent modification of enzymes and allosteric control. Based on the model, covalent modification is not strictly required. Hence, it is reasonable to question whether allosteric interaction alone between metabolites and enzymes can yield the properties of cyclic cascade systems, such as signal amplification, flexibility, and sensitivity with respect to increasing effector concentrations.

To accomplish signal amplification by means of simple allosteric control, the following conditions would have to be met: (i) very tight binding between the allosteric effector and the target enzyme, and (ii) a reaction which possesses catalytic properties such that one effector can activate more than one target enzyme molecule. Note that signal amplification in cyclic covalent modification cascades is achieved without a requirement for tight binding between effector and converter enzyme. Because the binding rate for the effector is limited by the diffusion rate, a slow off-rate for the enzyme-bound effector would be required to achieve tight binding. However, tight binding would reduce the temporal efficiency of the control process. Furthermore, in order to achieve a catalytic effect in a simple allosteric model, the effector would first have to bind to the target enzyme, induce an active conformation, and then dissociate from the active enzyme which would have to remain in the active conformation. Such a mechanism has been implicated in the past. However, to remain regulatable by the effector, the active enzyme would have to be able to relax back to its inactive form. This kind of mechanism is thermodynamically unfavorable. In addition, without the presence of converter enzymes, the capacity for allosteric interactions would be reduced considerably. Nevertheless, the apparent cooperativity which provides the sensitivity observed in cyclic cascade systems can be accomplished by allosteric interaction alone, particularly if the enzyme involved contains multiple subunits. In essence, some of the advantages derived from cyclic cascade regulation cannot be achieved without invoking reversible covalent modification, while others can be accomplished but with less regulatory efficiency.

IV. Protein-DNA Interactions

A. Kinetics of CRO Protein Binding to Specific and Nonspecific DNA

CRO protein, which binds to the operator site of the gene of lambda repressor protein and inhibits its synthesis, is used to study protein-DNA interaction. Upon DNA binding, whether it is specific or nonspecific, the tyrosine fluorescence is quenched significantly. Preliminary kinetic studies using the stopped-flow method show that the formation of the initial CRO-DNA complex, which is accompanied by a large fluoresence quenching, is too rapid for detection. Nevertheless, following the initial complex formation, the protein undergoes a relatively slow conformational change step. These stepwise reactions are observed for both specific and nonspecific DNA. However, the conformational change step induced by the specific DNA causes a further reduction in fluorescence intensity, while the nonspecific DNA causes an increase in fluorescence intensity. This

observation suggests that the extra stability for the CRO-specific DNA complex may derive from the pi-pi interaction between the base of DNA and the tyrosine residue of the protein.

V. Oxidative Degradation of Proteins.

It was found that storage of yeast glutamine synthetase (GS) in a buffer containing DTT causes the enzyme to degrade gradually. The degradation is not caused by contaminating proteases since it cannot be inhibited by the addition of various protease inhibitors. Further study revealed that the degradation required oxygen, one reducing equivalent of DTT, or ascorbate and a catalytic amount of iron or copper, a condition which is known to generate reactive oxygen species such as hydroxyl radical. It is believed that the degradation is caused by the formation of H_2O_2 due to oxygen reaction with the reducing agent such as ascorbate or DTT. H_2O_2 will then reduce the protein bound Fe^{3+} or Cu^{2+} and generate a superoxide, $O_2^-\cdot$. The reduced protein bound metal ion can then react with another H_2O_2 to form either Fe^{3+} or Cu^{2+} and hydroxyl radical which can oxidize a nearby amino acid residue in the enzyme and cause its degradation. It should also be pointed out that superoxide can also reduce either Fe^{3+} or Cu^{2+} to Fe^{2+} or Cu^{+}, respectively. In view of these possible reactions, only a catalytic amount of iron or copper is needed for this oxidative degradation to occur. In accord with the above interpretation, it was shown that this degradation was prevented by the addition of EDTA or Chelex treatment of all chemical reagents used. Several other proteins, namely, adenylyltransferase, E. coli GS and rabbit muscle pyruvate kinase have been shown to undergo similar oxidative degradation rapidly. However, cleavage of bovine serum albumin, ovalbumin, and A. niger glucose oxidase occurs slowly, and rabbit muscle aldolase is completely resistant to this degradation. In view of this finding, caution must be taken to prevent this oxidative modification when an enzyme is exposed to DTT since many biochemicals are contaminated with metal salts in sufficient quantities for catalyzing the formation of hydroxyl radicals.

Annual Report
Section on Protein Chemistry
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1985

Research in the Section on Protein Chemistry consists of studies on the physical and chemical properties of macromolecules of biological interest and on the roles of ligand binding and of protein-protein and inter- and intra-subunit interactions in enzyme catalysis and regulation. The energetics of ligand binding to proteins involves contributions from both ligand-protein and protein-protein interactions. Ligand promoted changes in protein-protein interactions underlie the phenomenon of cooperativity in ligand binding to proteins and, in addition, give rise to the numerous examples of stabilization and destabilization of protein structures by ligands, metal ions, and other inorganic ions.

Glutamine synthetase, a strictly regulated enzyme in Escherichia coli, is a dodecamer; each subunit (50,000 M_r) contains a catalytic site with two essential divalent cation sites (n_1 and n_2) and a tyrosyl residue that is the site of covalent modification by enzymatically-catalyzed adenylylation-deadenylylation reactions. The 12 identical subunits of enzyme are arranged in 2 superimposed hexagonal rings of about 140 Å in diameter and centers of adjacent subunits are ~ 45 Å apart. Studies of the interactions of divalent cations, substrates, substrate analogs, and inhibitors with glutamine synthetase from E. coli have continued.

We have shown that the very tight binding of 2 Mn^{2+}, L-methionine-S-sulfoximine phosphate, and ADP ($K_A' > 10^{12}\ M^{-1}$) formed on each subunit of E. coli glutamine synthetase at pH 7 by phosphorylation of the L-glutamate analog by ATP, stabilizes intersubunit bonding domains. Various analogs of ATP that are substituted at the 6- or 8-position of the adenine ring have since been shown to serve as substrates for the phosphorylation of L-met-S-sulfoximine and thereby can be introduced specifically into active-sites of the enzyme as structural probes. The distance between active sites of the enzyme has been measured by fluorescence energy transfer taking advantage of the essentially irreversible binding of various ADP analogs at neutral pH when bound with L-met-S-sulfoximine phosphate and Mn^{2+} at active sites. We used two fluorescent donors, either 8-mercapto ATP alkylated with N-(iodoacetylaminoethyl)-5-napthylamine-1-sulfonic acid (AEDANS-ATP) or 1-N^6-etheno-2-aza-ATP (aza-ε-ATP) and two acceptors, 6-mercapto purine ribonucleoside triphosphate or 8-mercapto ATP alkylated with the chromophore 4(p-dimethylaminophenylazo)phenyl-4-iodoacetamide. The fluorescence yields of enzyme derivatives with 1 or 2 eq of fluorescent donor per dodecamer and either an acceptor or ADP at the remaining active sites were compared at pH 7.0. Excellent agreement was obtained with the different combinations of donor/acceptor probes on the dodecameric enzyme, resulting in a maximum range of ± 2 Å in calculated distances between active-site probes. The results, together with the known geometry of the enzyme, indicate that active-site probes are widely separated and that energy transfer occurs from a single donor to 2 or 3 acceptors on adjacent subunits. The calculated distance between equidistant active-site probes on heterologously bonded subunits within the same hexagonal ring is 56-61 Å. Probes on isologously bonded subunits can be no closer than 60 Å and may be as far apart as 78 Å. Thus, active sites are away from the 6-fold axis of symmetry toward the outer edges of the dodecamer and are located

> 30 Å from the plane separating hexagonal rings.

We began this study on fluorescence energy transfer distances between active sites to determine whether the kinetic and binding data that indicated some form of communication between active sites of glutamine synthetase could be explained by the proximity of pairs of active sites in the dodecamer. The stabilization of submolecular oligomers by active-site ligands implies that ligands binding at the active site alter the structure of the intersubunit bonding domains. These conformational changes may also extend to adjacent active sites leading to enhanced binding of inactivating ligands and thus to a nonrandom distribution of inactive subunits in partially inactivated dodecamers. However, our results show that no two active sites of glutamine synthetase are closer to each other than to the rest of the active sites. Rather, it appears that the active-site nucleotide probes are arranged symmetrically within 4 Å of being at the maximum distance apart and that nucleotides at active sites of the heterologously bonded subunits are equidistant (~ 56-70 Å) from each other. Active-site probes of the subunits in the opposite ring are > 60 Å apart and located on the outer surface of the dodecamer away from the central 6-fold axis of symmetry. The location of the active sites with respect to the subunit bonding domains is under investigation but our results suggest that the nucleotide probes are near (± 5 Å) the center line of the subunits equidistant from the subunit contacts.

The same fluorescent enzyme derivatives that were used for the determination of intramolecular fluorescence energy transfer distances could be induced by Zn^{2+} in the presence of $MgCl_2$ to form face-to-face aggregates of enzyme dodecamers along the 6-fold axes of symmetry. The Zn^{2+}-induced stacking of glutamine synthetase dodecamers also could be fully reversed by adding a Zn^{2+} chelator such as EDTA. The kinetics of the Zn^{2+}-induced stacking reaction was measured by time-dependent fluorescence and light scattering changes; the fluorescence quench was dependent on the presence of acceptors in layered dodecamers and correlated well with the degree of linear polymer formation as a function of time. The time-dependent fluorescence quench during Zn^{2+}-induced face-to-face aggregation at pH 7.0 and 25°C had a second-order rate constant of ~ 10^5 s^{-1} M^{-1} at early stages, pseudo-first-order kinetics with half-times of 135-365s (depending upon the fluorescent probe attached) at later stages, and reached a maximum when the average n-mer was 6 dodecamers. With AEDANS-ADP, but not with aza-εADP, there was evidence of an intramolecular isomerization during the stacking process that may relate to the flexibility of the fluorescence probe. Thus, the approaches used in these studies also may be useful in studying the kinetics of other self-assembly systems when both a fluorescent donor and an acceptor are attached to the monomer species. In addition, for the face-to-face aggregation of dodecameric glutamine synthetase, an Arrhenius activation energy of 22.3 ± 0.2 kcal/mol was measured in the presence of 46 μM Zn^{2+} and 9.2 mM $MgCl_2$ at pH 7.0. This is a rather small activation energy, considering that Zn^{2+} binding produces a deformation of the enzyme and that 6-intermolecular subunit contacts must be formed in the stacking process. Moreover, the maximum quench obtained by stacking fluorescent derivatives of glutamine synthetase indicated that the average intermolecular distance between donor and acceptor probes in layered dodecamers is ~ 36 Å. This intermolecular energy transfer distance confirms that active-site nucleotide probes are toward exterior surfaces away from the lateral plane between hexagonal rings of the dodecamer.

We have found that mercapto nucleotides can form very stable complexes with aquo glycyl-L-methionato·Pt(II) and these have been used for introducing an

electron dense probe into active sites of glutamine synthetase. Also, we have found that we can adenylylate the enzyme using 6-S-ATP as a substrate of adenylyltransferase and then react the attached 6-S-AMP groups with the Pt(II) complex. These enzyme derivatives have been supplied to the Brookhaven National Laboratory (headed by J. S. Wall and J. F. Hainfeld) for scanning transmission electron microscopy (STEM) and computer imaging and to David Eisenberg at UCLA for X-ray crystallographic analysis. It now appears that the STEM analysis requires further refinements before an electron dense marker such as Pt(II) can be detected. Also, there have been problems in obtaining the correct crystal form of Pt(II)-enzyme derivative for X-ray crystallographic analysis. Nevertheless, we are persisting in these studies since an electron dense probe at active sites or at adenylylation sites potentially can solve important aspects of the 3-dimensional structure of glutamine synthetase.

Active-site ligand and metal ion interactions with mammalian octameric glutamine synthetase from bovine brain are being studied. The evidence obtained from binding and kinetic studies suggests that the enzyme has two essential metal ion binding sites per subunit, both of which must be filled for activity expression. The second Mn^{2+} binding site binds the nucleotide-metal ion complex ($K_A \simeq 10^6\ M^{-1}$) after the first site ($K_A \simeq 1.5 \times 10^5\ M^{-1}$) is occupied by Mn^{2+}. Filling the first site with Mn^{2+} or Mg^{2+} produces structural changes in the enzyme as evidenced by UV difference spectra and tryptophanyl residue fluorescence changes. Filling the second site requires the presence of nucleotide. Moreover, a Job analysis showed that all subunits of the bovine brain enzyme express γ-glutamyl transfer activity upon binding 1.0 eq of Mn•ADP complex per subunit. The affinity of the enzyme subunit for Mn•ADP is increased ~ 53-fold by the binding of arsenate or P_i (assay conditions). Although some of the features of Mn^{2+} binding of the brain enzyme are similar to those previously found for glutamine synthetase from E. coli, there are subtle differences. Furthermore, a specific binding of chloride ions to the brain enzyme ($K_A \simeq 8 \times 10^3\ M^{-1}$) has been found to destabilize this enzyme and to promote both fluorescence and UV absorbance changes. The effects of chloride ions on brain glutamine synthetase may be physiologically important.

The metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) has been used at pH 7.0 to monitor the mercurial-promoted Zn^{2+} release from E. coli aspartate transcarbamoylase and Zn^{2+} uptake by regulatory dimers upon displacement of the mercurial reagent with 2-mercaptoethanol. The release of Zn^{2+} (as reflected by a yellow to orange color change in PAR solutions) is linked to dissociation of the enzyme since six Zn^{2+} bonding domains stabilize catalytic and regulatory chain contacts; the rebinding of Zn^{2+} produces enzyme assembly and a corresponding decrease in the amount of PAR-Zn^{2+} complex. Using > 10-fold PAR to free Zn^{2+} at pH 7.0, $\Delta\varepsilon = 6.6 \pm 0.2 \times 10^4\ M^{-1}\ cm^{-1}$ at 500 nm (20°C) for $(PAR)_2Zn^{2+}$ complex formation ($\beta_2 \simeq 10^{12}\ M^{-1}$). In kinetic studies at pH 7.0, PAR (10^{-4} M) has been used to measure the instantaneous concentration of Zn^{2+} released from micromolar quantities of protein; second-order rate constant is $2 \times 10^7\ M^{-1}\ s^{-1}$ for forming the 1:1 PAR•Zn^{2+} complex. These properties of PAR-Zn^{2+} interactions make PAR a generally useful reagent for studying Zn^{2+} release from proteins. Current studies on the binding of Zn^{2+} to isolated regulatory subunits relate directly to the mechanisms of ATCase assembly in vivo.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00201-14 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Metabolism of the Branched-Chain Amino Acids

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

J. Michael Poston, Research Chemist, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.95	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A study of the metabolism of the branched-chain amino acids has revealed a pathway of metabolism of leucine that is catabolic in bacteria and appears to be synthetic in humans. The pathway depends upon the activity of the enzyme, leucine 2,3-aminomutase, an enzyme dependent upon adenosylcobalamin as a cofactor. Other enzymes which function in the pathway are β-leucine transaminase/deaminase, coenzyme A transferase, and thiolase. The relative carbon flux through this pathway and the pathway which is independent of cobalamin greatly favors the independent pathway in brain, heart, kidney, and liver. In the testis, however, the cobalamin-dependent pathway accounts for over forty percent of the carbon flux. This suggests that the metabolism of leucine may play an important role in this organ. The nature of the transaminase/deaminase will be examined and purification of the enzyme will be attempted. The relationship between enzyme activity and various disease states such as pernicious anemia and inborn errors of metabolism will be examined.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives: The catabolism of the branched-chain amino acids--leucine, isoleucine, and valine--remains incompletely understood. Although much information that is available has been derived from the study of inborn errors of amino acid metabolism, studies of bacterial fermentation of the amino acids and examinations of animal tissues have supplied additional information about the metabolism of these amino acids. The objectives of this project are to establish the fermentation pathways of leucine and the other branched-chain amino acids, to examine the enzymes responsible for the various metabolic steps in these fermentations, to explore the distribution of these pathways in other species, to examine the enzymes in these other species, and to examine the implications of these pathways in human metabolism.

Major Findings:

As previously reported, when cells or extracts of several clostridia, especially Clostridium sporogenes and Clostridium lentoputrescens, are incubated with L-leucine, several metabolic products are formed that are consistent with the metabolic pathway reported in mammals. However, the production of isobutyrate could not be explained by previously postulated pathways. Evidence was accumulated to support the pathway:

$$\alpha\text{-leucine} \rightleftharpoons \beta\text{-leucine} \rightleftharpoons \beta\text{-ketoisocaproate} \rightleftharpoons \text{acetate} + \text{isobutyrate}$$

This pathway, however, has been shown to be incomplete since the β-ketoisocaproate is not cleaved directly, but first must be converted to the coenzyme A thioester. As reported previously, the enzymes which carry out the conversion and the subsequent cleavage, coenzyme A transferase and thiolase, have been purified and have been shown to be the already well-characterized enzymes.

The first step in the pathway is catalyzed by leucine 2,3-aminomutase, an enzyme which has been shown to depend upon coenzyme B_{12} [adenosylcob(III)-alamin]. Although this enzyme is quite stable in crude extracts and in only partially purified states, it has proven to be quite easily lost when it has been purified about twenty fold. Nonetheless, it has been demonstrated to be widely distributed in nature, being found in micro-organisms, plants, animals, and humans.

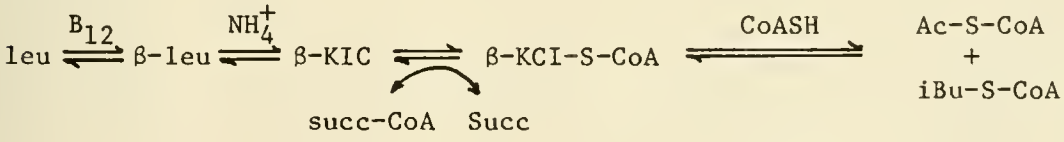
The second step has been assumed to be a transaminase. There is now evidence to suggest that it may be more similar to a deaminase instead. When coenzyme A esters of radiolabelled acetate and isobutyrate are incubated with crude extracts of rat liver or kidney, label is incorporated in both β-leucine and leucine. This incorporation is stimulated by the addition of ammonium ion and is strikingly inhibited by the presence of glutamate or of alanine, common participants in transamination reactions. Moreover, both reduced pyridine nucleotide (NADH or NADPH) and ATP inhibit this incorporation. This suggests that the conversion of the β-ketoisocaproate formed from the two thioesters to a cationic form must involve a direct uptake of an amino group without involvement of a reductant. Alternatively, of course, it may be that

the reduced pyridine nucleotide serves as a potent effector and the apparent inhibition of the incorporation is a consequence of allosteric interactions. In any event, these findings explain some of the difficulty in attempting to demonstrate the existence of the enyzme which catalyzes the conversion of β-leucine to β-ketoisocaproate. The very compounds which were added to reactions to demonstrate transaminations served to inhibit the reactions.

However, evidence supporting the transaminase-like character of the second step is shown in the response to cycloserine. This inhibitor is known to be a potent inhibitor of transaminases and, when reactions were conducted in the presence of cycloserine, little radiolabel from labelled acetyl-S-CoA was incorporated into either β-leucine or leucine. There is little effect of added pyridoxal phosphate.

When radiolabelled β-ketoisocaproate was incubated with extracts of rat liver, little or no radioactivity could be recovered in the cationic form. This finding was very discouraging, but now that the inhibitory nature of glutamate, NADH, and ATP has been shown, these experiments will be repeated with ammonium ion. Experiments with the labelled β-ketoisocaproate are extremely difficult because the substrate has such a low specific activity. There is continuing concern that the low counts detected may lead to errors in interpretation. The labelled β-ketoisocaproate was synthesized by Dr. Lin Tsai of this laboratory. He has predicted that it may be possible to increase the specific activity of the substrate 2-5 fold. If that is possible, it may be that the reactions can be characterized with more assurance. One of the chemical problems with this compound is that, as a β-keto acid, it is subject to decarboxylation under fairly mild conditions. The product, methyl isopropyl ketone, is often found distributed inconsistently between the cationic and anionic fractions.

When non-labelled β-ketoisocaproate is incubated with cell-free extracts of rat kidney, there is a substrate-dependent production of β-leucine and of leucine. When the mucoprotein, intrinsic factor, is added to the reaction mixtures, it binds coenzyme B_{12} very tightly and effectively removes it from use by the leucine 2,3-aminomutase. Consequently, there appears to be little or no leucine produced under these conditions. This is consistent with the pathway:



Because, as has been previously reported, the nature of this pathway seems to be a means of biosynthesis of leucine it may be that there are inborn errors in some individuals which prevent them from synthesizing any leucine. Under conditions of complete nutrition, such a deficit may pose no problem; but, if the individual may be otherwise limited in leucine, a faulty biosynthetic pathway may bring the individual into negative nitrogen balance. There has been a continuing survey of sera from patients around the world in the hope that a serum leucine and β-leucine abnormality might be correlated

with some disease state. To date, however, the only clearcut correlation is with patients who have untreated pernicious anemia. Because of the blockage of leucine 2,3-aminomutase in that disease of B_{12} deficiency, β-leucine cannot be converted to leucine and circulating levels of β-leucine may be elevated.

Significance to Biomedical Research and the Program of the Institute:

This study impinges on several areas of medical concern: the mode of action of vitamin B_{12} in its metabolic roles, the metabolism of amino acids, and nutrition. The mode of action of B_{12} is imperfectly understood, but its importance in hematopoiesis and in the maintenance of proper neurological function is exemplified in the disease of its metabolic deficiency, pernicious anemia. Prior to these studies, only two B_{12}-dependent enzymes had been demonstrated in man; leucine 2,3-aminomutase is now the third. The finding that the endocrine tissue of rats has a strong metabolism in the B_{12}-dependent pathway may have significance in the understanding of some human metabolic problems. Several inborn errors have been involved with amino acid metabolism and the effects of these errors may be devastating to the well being of humans, especially in the instances of maple syrup urine disease, isovaleric-acidemia, and disorders of the catabolism of short-chain acids. The β-leucine pathway may be involved in some of the syndromes associated with these inborn errors.

Proposed Course:

The enzymes of the β-leucine pathway will be further purified and characterized. Special effort will be given to the purification of the transaminase and the aminomutase. The relationship of the metabolism of leucine via the β-leucine pathway to the endocrine tissue of the testis will be examined.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00202-14 LB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Kinetics, Regulation and Mechanism of Biochemical Reactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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SECTION
Section on Metabolic Regulation

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.4	3.0	0.4

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

(1) An improved purification procedure using the HPLC technique has been developed to purify the Mg(II)-ATP-dependent protein kinase from rabbit skeletal muscle. The purified enzyme consists of 38 and 31 KDa subunits, which correspond to the catalytic and modulator subunit, respectively. A mechanism for the activation of the inactive enzyme involves a transient phosphorylation of the modulator subunit catalyzed by kinase Fa. A few lines of evidence in support of the phosphorylation induces the catalytic subunit to its active form includes the observation that removal of the modulator by limiting proteolysis fails to activate the inactive (native) enzyme, unless it has been first phosphorylated or thiophosphorylated. Evidence supporting the slow isomerization which leads the active enzyme to its inactive form was demonstrated. The regulatory subunit (R) of type II cAMP-dependent protein kinase is an effective inhibitor for the phosphatase. A curve fitting method was used to estimate the dissociation constants for R•phosphatase, R•type I catalytic fragment and Fa•phosphatase complexes. The results suggest that R binds to the catalytic subunit. In addition, computer simulation of a cyclic cascade using the cAMP-dependent protein kinase and the above phosphatase as converter enzymes revealed that they constitute a highly efficient regulatory mechanism.

(2) A comparative study of cyclic cascade and simple allosteric control revealed that simple allosteric control cannot provide the same regulatory efficiency as those exhibited by the cyclic cascade systems.

(3) A simple treatment was developed to describe interfacial reaction dynamics. This analysis revealed those conditions under which reduced dimensionality can be expected to enhance the overall efficiency or reaction rate of a bimolecular reaction.

(4) A study of skeletal muscle actomyosin ATPase cycle using subfragment-1 in place of myosin revealed that a six-state model, which consists of six myosin bound reaction intermediates containing phosphate in the form of ATP or ADP•Pi, is the simplest kinetic model required to describe the ATase cycle.

45

Project Description:

Objectives:

(1) Utilizing the physical-chemical approach to study the kinetics, mechanism, and regulation of biochemical activity. In particular, the biochemical action between the substrates, metabolites, and enzymatic activity of glutamine synthetase from Escherichia coli will be elucidated. In addition, fast kinetic techniques which are useful in these studies will be improved.

(2) Theoretical analysis of the cyclic cascade system with respect to its properties and function in the metabolic regulation of key enzymes will be continued.

(3) Isolation of the regulatory proteins to allow detailed mechanistic studies, and experimentally verify the validity of the cyclic cascade model described in (2).

(4) To study the regulation of Mg(II)-ATP-dependent protein phosphatase.

Major Findings:

1. Regulation of the Mg(II)-ATP-dependent Protein Phosphatase (also see S. Jurgenson's Annual Report). The Mg(II)-ATP-dependent protein phosphatase, inactive as isolated, is a major phosphorylase phosphatase in skeletal muscle and has been found in numerous other tissues. An improved purification procedure has been developed in which TSK-DEAE HPLC column chromatography and TSK-phenyl HPLC column chromatography steps are used. The TSK-DEAE HPLC column step separates a 62 KD_a polypeptide which had previously copurified with enzyme activity and with the 38 and 31 KD_a subunits. However, this chromatographic step failed to remove a 68-70 KD_a protein which can be removed by the TSK-phenyl HPLC chromatographic step. The highly purified enzyme consists of 38 and 31 KD_a subunits. The specific activity of this enzyme is protein concentration dependent. In dilute solution (~ 0.1 mM), the specific activity is 13,000 nmole/min/mg. This value is decreased to about 3,500 nmole/min/mg at high protein concentration (~ 6 nM). These data suggest an association/diassociation mechanism which affects the activity of the activated enzyme.

The activation mechanism involves a transient phosphorylation of the 31 KD_a subunit (also known as modulator or inhibitor-2) catalyzed by kinase F_A (also known as glycogen synthase kinase-3). Phosphorylation of the modulator causes a conformational change in the 38 KD_a catalytic subunit, and leads to the activation of the phosphatase. The active phosphatase is capable of dephosphorylating the phosphorylated modulator causing the phosphatase to return to its inactive form via a slow isomerization step. The time course of the phosphatase activation and of phosphate incorporation into the modulator subunit was investigated. The phosphorylation is rapid, and as phosphatase activation proceeds, there is a decrease in the amount of phosphate incorporation before it reaches a steady state level. A couple lines of evidence in support of the phosphorylation induces a conformational change of the catalytic subunits such that it converts from its inactive to its active form are: (i) removal of the modulator subunit by limiting proteolysis fails to activate the native (inactive) phosphatase

unless it has been first phosphorylated; (ii) the modulator can be thiophosphorylated, however, it fails to activate the enzyme. Upon removal of modulator by limiting proteolysis, the enzyme becomes activated. The slow isomerization step which leads the active enzyme to its inactive form was demonstrated by blocking the activation reaction with EDTA and then following the time course of the inactivation reaction. This reaction is first-order with a $t_{1/2}$ equals to 13-15 minutes.

The inhibition of the Mg(II)-ATP-dependent protein phosphatase by the regulatory subunits of type II cAMP-dependent protein kinase was further investigated. Analysis of the inhibition data, with the assumption that the regulatory subunit binds on a single site on the phosphatase yields apparent K_D of about 100 and 42 nM for reaction mixture containing 400 ng/ml and 80 ng/ml of F_A, respectively. This suggests an antagonism between the binding of regulatory subunit and of kinase F_A to the phosphatase. If one assumed a mutually exclusive mechanism for these bindings, one can estimate a K_D value of ~ 3 nM for F_A binding to phosphatase and K_D for regulatory subunit•phosphatase complex is ~ 24 nM which is quite similar to the K_i (15 nM) for regulatory subunit inhibition of the catalytic fragment of the phosphatase. This seems to support the hypothesis that the regulatory subunit binds to the phosphatase instead of the kiase F_A. In addition, computer simulation of a cyclic cascade system using the cAMP-dependent protein kinase and the Mg(II)-ATP-dependent protein phosphatase as two converter enzymes reveals that they constitute a highly coordinated regulatory cascade. This synchronous regulatory cascade provides additional signal amplification and it enhances the sensitivity in changes of fractional phosphorylation of the interconvertible enzyme in response to increasing cAMP concentration. In addition, the coincident activation of protein kinase and inhibition of protein phosphatase reduces the ATP consumption required for maintaining the function of a cyclic cascade, thus making it more energetically efficient.

2. Cyclic Cascade vs Simple Allosteric Control. Metabolic regulation by a cyclic cascade model utilizes both covalent modification of enzymes and allosteric control. Based on the model, covalent modification is not strictly required. Hence, it is reasonable to question whether allosteric interaction alone between metabolites and enzymes can yield the properties of cyclic cascade systems, such as signal amplification, flexibility, and sensitivity with respect to increasing effector concentrations.

To accomplish signal amplification by means of simple allosteric control, the following conditions would have to be met: (i) very tight binding between the allosteric effector and the target enzyme, and (ii) a reaction which possesses catalytic properties such that one effector can activate more than one target enzyme molecule. Note that signal amplification in cyclic covalent modification cascades is achieved without a requirement for tight binding between effector and converter enzyme. Because the binding rate for the effector is limited by the diffusion rate, a slow off-rate for the enzyme-bound effector would be required to achieve tight binding. However, tight binding would reduce the temporal efficiency of the control process. Furthermore, in order to achieve a catalytic effect in a simple allosteric model, the effector would first have to bind to the target enzyme, induce an active conformation, and then dissociate from the active enzyme which would have to remain in the active conformation. Such a mechanism has been implicated in the past. However, to remain regulatable

by the effector, the active enzyme would have to be able to relax back to its inactive form. This kind of mechanism is thermodynamically unfavorable. In addition, without the presence of converter enzymes, the capacity for allosteric interactions would be reduced considerably. Nevertheless, the apparent cooperativity which provides the sensitivity observed in cyclic cascade systems can be accomplished by allosteric interaction alone, particularly if the enzyme involved contains multiple subunits. In essence, some of the advantages derived from cyclic cascade regulation cannot be achieved without invoking reversible covalent modification, while others can be accomplished but with less regulatory efficiency.

3. Kinetics of CRO Protein Binding to Specific and Nonspecific DNA (also see R. D. Astumian's Annual Report). CRO protein, which binds to the operator site of the gene of lambda repressor protein and inhibits its synthesis, is used to study protein-DNA interaction. Upon DNA binding, whether it is specific or nonspecific, the tyrosine fluorescence is quenched significantly. Preliminary kinetic studies using the stoppedflow method show that the formation of the initial CRO-DNA complex, which is accompanied by a large fluoresence quenching, is too rapid for detection. Nevertheless, following the initial complex formation, the protein undergoes a relatively slow conformational change step. These stepwise reactions are observed for both specific and nonspecific DNA. However, the conformational change step induced by the specific DNA causes a further reduction in fluorescence intensity, while the nonspecific DNA causes an increase in fluorescence intensity. This observation suggests that the extra stability for the CRO-specific DNA complex may derive from the pi-pi interaction between the base of DNA and the tyrosine residue of the protein.

4. Theoretical Treatment of Interfacial Reaction Dynamics (also see R. D. Astumian's Annual Report). Biomolecular reactions in which one of the reactants is localized at an interface while the other reactant is initially molecularly dispersed in the homogenous phase can occur by two paths. One involves the direct interaction of a homogenous reactant with its interfacially localized reaction partner, and the other proceeds by initial adsorption of the homogenous reactant and subsequent surface diffusion to reaction. A branching method for the treatment of the surface dynamics was developed whereby the total reaction velocity is given by the rate at which reactants enter into a specified, arbitrary configuration, multiplied by the product of the probabilities of the subsequent individual event necessary for reaction to occur. This treatment is particularly useful for the analysis of reactions which occur by multiple mechanisms. Theoretical analysis shows that although rate enhancement as compared to an analogous homogenous reaction (with three-dimensional diffusion) can be anticipated only for very low interfacial concentrations of localized reactant, the two-dimensional surface diffusion mechanisms accounts for an appreciable portion of the total reactivity within a wide range of circumstances for ligand-cell surface receptor interactions. In addition, this study revealed that in systems with many reactive sites localized on a single moiety, e.g., a single polypeptide, the diffusion controlled binding kinetics of ligand to those sites will be nonlinear, thus, apparent kinetic cooperative effects can be observed, even in the absence of site-site interactions.

5. Mechanistic Study of Rabbit Skeletal Muscle Actomyosin ATPase Cycle (also see E. Eisenberg's Annual Report). Muscle contraction is driven by a cyclic reaction between actin and myosin, coupled to the hydrolysis of ATP. Two

models have been proposed to account for the kinetic data of ATP hydrolysis. A four-state model which has four states containing bound ATP or ADP•P_i and the rate-limiting step is ATP hydrolysis. In the six-state model, there are six myosin bound reaction intermediates containing phosphate in the form of ATP or ADP-P_i, and the rate-limiting step is a conformational change which occurs before P_i release but after ATP hydrolysis. A difference between these two models is that only the four-state model predicts that almost no acto-subfragment 1•ADP•P_i (acto-S-1•ADP•P_i) complex will be formed when ATP is mixed with acto•S-1. Results from investigating the amount of acto•S-1•ADP•P_i formed when ATP is mixed with S-1 cross-linked to actin suggests that at both high and low ionic strength the ATP hydrolysis step is not the rate-limiting step. Instead, the data are consistent with the rate-limiting step occurring before P_i release and after ATP hydrolysis step, as proposed in the six-state kinetic model.

Significance to Biomedical Research and the Program of the Institute:

The overall objective is to gain a better understanding of how enzymes function with respect to their catalytic and regulatory properties, and to elucidate principles of interaction between effectors, regulators, and proteins. This knowledge is instrumental in controlling the function of a specific enzyme by designing an effector or enzyme suicide substrate.

Proposed Course:

(1) To further expand the cyclic cascade model to substrate cascade and more complex enzyme cascade systems; and to further analyze the kinetic aspect of the cyclic cascade.

(2) To study the mechanism of the cascade reactions which regulate the state of adenylylation for glutamine synthetase. We plan to study the protein-protein interaction between P_{II} protein and ATase, and UTase, and ATase-glutamine synthetase interaction, by physical, chemical, and immunological methods. To test the validity of the bicyclic cascade model, and to study the role of the effectors in this cascade system.

(3) To further explore the physical, chemical, and immunological properties of unadenylylated and adenylylated glutamine synthetase. In particular, we will utilize the fast reaction techniques, NMR, ESR, and fluorescence polarization methods to elucidate the roles of effectors and to identify the intermediates in the catalytic cycle. Laser light scattering will be used to study protein-protein interactions.

(4) To investigate the activation mechanism of cAMP-dependent protein kinase and reaction mechanism of phosphoprotein phosphatase.

Publications

Shacter, E., Chock, P. B., and Stadtman, E. R.: Regulation through phosphorylation/dephosphorylation cascade system. J. Biol. Chem. 259: 12252-12259, 1984.

Shacter, E., Chock, P. B., and Stadtman, E. R.: Energy consumption in a cyclic phosphorylation/dephosphorylation cascade. J. Biol. Chem. 259: 12260-12264, 1984.

Stein, L. A., Chock, P. B., and Eisenberg, E.: The rate-limiting step in the actomyosin adenosine triphosphatase cycle. Biochemistry 23: 1555-1563, 1984.

Rhee, S. G., Chock, P. B., and Stadtman, E. R.: Nucleotidylation involved in the regulation of glutamine synthetase in E. coli. In Freedman, R. (Ed.): The Enzymology of Posttranslational Modification of Proteins. New York, Academic Press, Vol. II, 1984, pp. 273-297.

Rhee, S. G., Chock, P. B., and Stadtman, E. R.: E. coli glutamine synthetase. Meth. Enzymol. 103: 213-241, 1985.

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Vandenheede, J. R., Yang, S. D., Merlevede, W., Jurgensen, S. R., and Chock, P. B.: Kinase F_A-mediated regulation of rabbit skeletal muscle protein phosphatase: Reversible phosphorylation of the modulator subunit. J. Biol. Chem. 260, in press, 1985.

Chock, P. B., Shacter, S., Jurgensen, S. R., and Rhee, S. G.: Cyclic cascade systems in metabolic regulation. Curr. Top. in Cell. Regul. 27, in press, 1985.

Shacter, E., Chock, P. B., Rhee, S. G., and Stadtman, E. R.: Cyclic cascades and metabolic regulation - Perspectives. In Krebs, E. and Boyer, P. D. (Eds.): The Enzymes, 3rd ed., Vol. 18, in press, 1985.

Stein, L. A., Greene, L. E., Chock, P. B., and Eisenberg, E.: Rate-limiting step in the actomyosin adenosine triphosphatase cycle: Studies with myosin subfragment-1 cross-linked to actin. Biochemistry 24, 1357-1363, 1985.

Shacter, E., McClure, J. A., Korn, E. D., and Chock, P. B.: Immunological characterization of phosphoprotein phosphatases. Arch. Biochem. Biophys., in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00203-12 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular Regulation of Enzyme Levels

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Cynthia N. Oliver, Staff Fellow, Laboratory of Biochemistry, NHLBI

Others: James Yan, Staff Fellow, Laboratory of Biochemistry, NHLBI
E. R. Stadtman, Chief, Laboratory of Biochemistry, NHLBI

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SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are actively studying the potential physiological role of oxidative modification of proteins. The current focus of this project concerns three areas, (1) neutrophil activation, (2) the role of protein kinase C in neutrophil activation, and (3) studies on a gliosis model system. Earlier studies have indicated that activated neutrophils can catalyze the oxidative modification of glutamine synthetase in intact E. coli as well the oxidative modification of endogenous neutrophil enzymes. These studies have shown that both the kinetics and the properties of the reaction are influenced by the activating agent (and/or the differentiating agent in the case of HL-60 cells). These results have suggested the presence of multiple pathways for neutrophil activation.

In order to elucidate the fundamental differences in these pathways, we have studied some reactions which appear to be relatively specific for a pathway activated by PMA. We present here evidence that tyrosine is incorporated into proteins of activated neutrophils by a mechanism which is independent of protein synthesis but is dependent on PMA-stimulated respiratory burst activity. In other studies we are examining the role of protein kinase C in neutrophil activation. We have demonstrated that several proteins are phosphorylated in a protein kinase C-dependent reaction following PMA activation. Finally, we are studying the oxidative modification of proteins in a culture system which is used for a model of gliosis.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives: This project involves continuing studies on the potential physiological role of oxidative inactivation of enzymes in biological systems. Although studies on aging model systems and oxidative modification of proteins in Alzheimer's disease have continued, our primary focus has been in three areas: (1) incorporation of tyrosine into proteins of activated neutrophils, (2) the role of protein kinase C in activated neutrophils, and (3) oxidative modification in gliosis.

Major Findings:

Tyrosine Incorporation in Activated Neutrophils

The process of bacterial enzyme inactivation may be functionally important in host defense against microbial infection in higher organisms. Mature polymorphonuclear leukocytes (neutrophils) are capable of ingesting and killing microorganisms. The cells can be activated chemically (as well as by microorganisms) by a variety of agents including bacterial chemotactic peptide (fMet-Leu-Phe, fMLP), latex beads, plant lectins, particles, complement, and phorbol ester (phorbol myristate acetate, PMA) to undergo an oxidative burst. This process is characterized by a dramatic change in oxygen uptake, a shift in metabolism from glycolysis to hexose monophosphate pathway, activation of NADPH oxidase, and the generation of a variety of activated oxygen species including O_2^-, OH, and H_2O_2. Studies have shown that activated oxygen species produced during the oxidative burst of neutrophils are diffusable and may be instrumental in bacterial target cell damage and death.

Two enzymes are thought to play a major role in the generation of activated oxygen species in activated neutrophils, a myeloperoxidase which is associated with neutrophil primary granules and a membrane-associated NADPH-oxidase which becomes activated during the oxidative burst. It is well known that patients with chronic granulomatous disease (CGD) ingest bacteria normally but exhibit a defective oxidative burst and often a deficient NADPH-oxidase activity. As a result, these patients suffer from recurrent microbial infections. Similarly, patients with hereditary myeloperoxidase deficiency kill some microorganisms much less efficiently than normal neutrophils. We have demonstrated that activated neutrophils can inactivate enzymes in intact bacteria as well as endogenous neutrophil enzymes. Because our previous results have suggested that differences in both the kinetics and the extent of inactivation are related to the pathway of activation, we have undertaken additional studies to elucidate the fundamental properties of these pathways.

In the course of studies undertaken to determine whether tyrosinolation of proteins in neutrophils occurred as a function of activation, Dr. Nath observed that large quantities of ^{14}C-labeled tyrosine were incorporated into TCA-precipitable material during activation. No incorporation of other labeled amino acids occurred under the same conditions and the incorporation of tyrosine could not be blocked by agents which inhibit protein synthesis. Moreover, incorporation occurred only when the cells were activated by PMA

but not by fMLP. Activated neutrophils from patients with chronic granulomatous disease (CGD) failed to exhibit this phenomenon but activated neutrophils from individuals with hereditary myeloperoxidase deficiency incorporated the labeled tyrosine. These results suggested that incorporation of the labeled tyrosine was associated with the pathway for NADPH-oxidase activation and was independent of protein synthesis.

We have previously demonstrated that activated neutrophils are capable of inactivating endogenous enzymes and generating protein carbonyl derivatives as determined by DNPH-reactivity. Because the labeled tyrosine incorporation was associated with PMA activation and oxidative burst, we have carried out studies to determine whether tyrosine incorporation has any effect on carbonyl generation. For these studies we have used karyogranuloplasts which are nuclear- and granule-enriched neutrophils prepared from leukophoresis leukopacks obtained from normal donors. Activation experiments were carried out in PMA in the presence or absence of added tyrosine (10^{-6} M). Cells incubated with PMA in the absence of tyrosine exhibit increased DNPH-reactivity compared to control cells (unactivated cells). Cells prepared from the same donor at the same time and incubated in the presence of added tyrosine (labeled or unlabeled) exhibited higher basal levels of DNPH-reactivity as well as a two-fold stimulation with PMA activation. It should be noted that higher basal levels of DNPH-reactivity are frequently observed in cells prepared by leukophoresis compared to non-leukophoresis cells.

Reverse phase HPLC analysis of the derivatized protein from experiments carried out in the presence of labeled tyrosine indicated that the label was present in multiple peaks and distributed throughout the protein (peptide) fractionation range. Moreover, the profiles of the samples incubated in the presence or absence of added tyrosine were nearly superimposable. Integration of the A375 signal indicated that the carbonyl content was indeed increased in the sample incubated with tyrosine, but the pattern was the same. Results of SDS gels also indicated that the protein patterns were identical . These observations suggest that although added tyrosine appears to potentiate the carbonyl generation during activation, no extensive change in protein cross linking occurs (which might be manifested by altered molecular weight distribution on SDS gels or increased hydrophobicity on reverse phase HPLC).

Foerder and Shapiro (Proc. Natl. Acad. Sci. U.S.A. 74, p. 4214, 1977) have described an ovoperoxidase-dependent formation of dityrosine cross links in the fertilization membrane following egg activation. Because egg activation and neutrophil activation have some similar properties, we have considered the possiblity that free tyrosine may undergo activation (possibly forming a tyrosine radical) in the presence of O_2^-, H_2O_2, or other activated oxygen species. This "activated" tyrosine may then (1) undergo some form of pheonolic coupling to tyrosines already in proteins or (2) react with other amino acids in proteins. We have compared the properties of tyrosine incorporation into proteins during neutrophil activation with the reaction characterized by Foerder and Shapiro. We found that incorporation was blocked by azide, phenylhydrazine, and sodium sulfite. These agents are known to be relatively nonspecific inhibitors of peroxidase-mediated reactions. The reaction is also

inhibited by N-acetyl-L-tyrosine and O-methyl-L-tyrosine. With the exception of O-methyl-L-tyrosine all of these agents inhibit hardening of the fertilization membrane. Foerder and Shapiro concluded from their studies with the tyrosine analogs that the amine function is not required for hardening, but the phenolic function is required. However, this may be dependent on the mechanism by which tyrosine is incorporated into proteins and whether protein cross linking occurs. We have measured the tyrosine pools in a single sample of neutrophils from a normal donor by amino acid analysis and the value obtained is 900 μM. The reported values for plasma, erythrocytes, and leukocytes are 70 μM, 80 μM, and 480 μM, respectively (McMenamy, R. P., 1969, J. Clin. Invest. 39:1675) using a paper chromatography method. In our experiments 1 μM tyrosine was added and it is not yet clear why the basal level of carbonyl generation should be increased in the presence of such a small amount of tyrosine. One possibility is that the addition of even small amounts of tyrosine leads to a new distribution or compartmentalization of tyrosine with respect to the membrane and the production of extracellular O_2^- and H_2O_2 via NADPH-oxidase at the membrane.

Protein Kinase C

It is now well established that PMA and other phorbol esters which are capable of activating neutrophils also activate a phospholipid and Ca^{++}-dependent protein kinase C. These observations have suggested that protein kinase C may be involved in neutrophils activation and that one or more phosphorylated products may be required for activation.

In an effort to identify C kinase dependent phosphorylated substrates, ^{32}p protein-labeling patterns were examined by SDS gel electrophoresis of neutrophil extracts prepared from PMA activated cells. Approximately eight protein bands ranging from 15kd-140kd were phosphorylated with $^{32}p\gamma$-ATP in the presence of phospholipid and Ca^{++} and two bands (40kd and 15kd) were especially prominent. Similar studies have been carried out with HL-60 cells. These cells can be differentiated by a variety of agents which give rise to different end stage type cells. DMSO and retinoic acid promote a myelocytic type of differentiation with the formation of a neutrophil-like cell. When cells are treated in culture with 1% DMSO for four to six days, respiratory burst activity can be detected as early as 24 hours. Moreover, during differentiation with DMSO, HL-60 cells exhibit a progressive increase in protein kinase C-dependent phosphorylation. Under these conditions again about eight protein bands are phosphorylated and again two bands (40kd and 15kd) were prominent. It is interesting to note that treatment of HL-60 cells with retinoic acid leads to little or no phospholipid and Ca^{++}-dependent phosphorylation and little or no respiratory burst competence as determined by cytochrome C reduction.

When HL-60 cells are treated with PMA they differentiate into a macrophage-like cell. Under these conditions a different phosphorylation pattern is observed, but again the 40kd band is prominent. Macrophages also exhibit respiratory burst activity, but it is usually of longer duration and lower intensity than that of neutrophils.

Gliosis

Some preliminary studies have been carried out with Dr. Halks-Miller of Stanford University. Dr. Halks-Miller is studying gliosis and has developed a model system using cultured brain cells. Under specified conditions dispersed brain cells undergo reorganization in vitro forming spheres containing a central core of neuronal elements and an outer layer of glial cells. Subsequent injury of the neuronal cells triggers growth and migration of the glial cells to the site of injury. Histological examination of the injured spheres reveals extensive accumulation of glial cells with concomitant damage to the neuronal cells. This process resembles the pathology of gliosis and glial scarring in vivo. Moreover, this process is accompanied by the production of activated oxygen species and the generation of malondialdehyde (presumably from lipid peroxidation). Both the extent of gliosis and the production of malondialdehyde is reduced in injured spheres treated with α-tocopherol. It was of interest to determine whether oxidative modification of proteins occurred under these conditions as determined by DNPH-reactivity. We found very high levels of DNPH-reactive material and these levels were not reduced by treatment with α-tocopherol. For these studies, the DNPH-reactive material was extracted extensively with organic solvents and under these conditions it is likely that most malondialdehyde derivatives from membrane lipids would be removed, although quantitative determinations were not carried out. However, it would not be surprising that α-tocopherol, which is a lipid soluble vitamin, would have a greater capacity to reduce peroxidation of membrane lipids than to reduce the oxidative modification of soluble intracellular proteins.

Proposed Course:

Tyrosine Incorporation in Activated Neutrophils

Subsequent investigations in this area will be concerned with two major questions; namely, (1) what is the nature of tyrosine incorporation into proteins of activated neutrophils and (2) what is the nature of the pathway which leads to tyrosine incorporation. Experiments are planned to carry out amino acid analysis of the protein hydrolysate from neutrophils activated with PMA in the presence of labeled tyrosine. By following the tyrosine label under these conditions it may be possible to determine whether tyrosine reacts randomly with multiple amino acids or with other tyrosines in proteins (possibly with the formation of dityrosine). It is likely that amino acid analysis may also provide some information about the pathway of incorporation and whether "activation" of the tyrosine is required for incorporation. The evidence to date suggests that tyrosine incorporation is dependent on activation of the NADPH-oxidase pathway and possibly the extracellular production of O_2^- or H_2O_2. If this is correct, then activation in the presence of fMLP and the calcium ionophore A23187 ought to lead to tyrosine incorporation. However, it is not yet clear why agents which are known to inhibit heme proteins and peroxidase-mediated reactions inhibit the incorporation of tyrosine via the NADPH-oxidase (flavoprotein) pathway unless an azide sensitive protein (possibly cytochrome b559) is an essential component for terminal

electron transport and tyrosine "activation." It may be possible to test this idea using neutrophils from individuals with hereditary myeloperoxidase deficiency. Moreover, these studies may elucidate some of the fundamental differences between the different pathways of neutrohpil activation as well as the nature of oxidative damage conferred on cells and tissues by activated neutrophils.

Protein Kinase C

Although not definitive, these results suggest that the phosphorylation of the 40kd band may correlate in a general way with respiratory burst competence and that this protein may be important in neutrophil activation or its regulation. Future studies will be directed toward the isolation and characterization of this protein. It is also of interest to determine whether other activating agents lead to the phosphorylation of this protein and whether this event is associated with the activation of NADPH-oxidase.

Gliosis

Time course experiments are planned in order to determine whether malondialdehyde and DNPH-reactive material are generated simultaneously. There is some evidence that glial cells have phagocytic activity in nervous tissue and it is possible that under some conditions these cells can be activated like neutrophils. Based on previous experiments with in vitro mixed-function oxidation systems, agents which potentiate or inhibit the oxidative modification of proteins will be tested to determine whether they have any effect on gliosis, malondialdehyde formation, and DNPH-reactivity in cultured spheres.

Publications:

Nakamura, K., Oliver, C., Stadtman, E.R. Inactivation of Glutamine Synthetase by a Purified Rabbit Liver Microsomal P_{450} System. Archives of Biochemistry and Biophysics 240: in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00204-18 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Protein Structure: Enzyme Action and Control

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ann Ginsburg	Chief, Section on Protein Chemistry	LB, NHLBI
Others:	Harold B. Pinkofsky	Staff Fellow (8/22/82-8/2/85)	LB, NHLBI
	Sue H. Neece	Chemist (part-time 3/16/82-11/23/84)	LB, NHLBI
	Marlana B. Blackburn	Staff Fellow (1/22/84-)	LB, NHLBI
	Philip G. Kasprzyk	Staff Fellow (6/24/84-5/3/85)	LB, NHLBI
	Patrick J. McFarland	Chemist (10/14/84-)	LB, NHLBI

COOPERATING UNITS *(if any)*

M.R. Maurizi, NCI, Lab. Molecular Biology; J. Hainfeld, J.S. Wall, and J.J. Lipka, Biological Dept., Brookhaven National Lab., N.Y.; J.B. Hunt, Catholic University of America; H.K. Schachman, University of California, Berkeley; D. Eisenberg, University of California, Los Angeles; J.R. Knutson, Lab. Technical Development, NHLBI

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Protein Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.3	5.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

(1) Nucleotide analogs have been introduced as structural probes into active sites of dodecameric glutamine synthetase from E. coli. Various analogs of ATP that are substituted at the 6- or 8-position of the purine ring have been further modified with spectrophotometric and fluorometric probes or an electron dense Pt(II) marker. Fluorescence energy transfer measurements were made with enzyme derivatives containing 1 or 2 eq of fluorescent nucleotide donor per dodecamer and ADP or acceptor analogs at the remaining active sites. Energy transfer was found to be from a single donor to 2 or 3 acceptors on adjacent subunits. The calculated distance between equidistant active-site probes on heterologously bonded subunits within the same ring is 56-61 Å. Probes on isologously bonded subunits are no closer than 60 Å and may be as far apart as 78 Å. Thus, active sites are away from the 6-fold axis of symmetry toward the outer edges of the dodecamer and are located > 30 Å from the plane separating the hexagonal rings. The same fluorescent enzyme derivatives were used also to study the kinetics of zinc-induced face-to-face aggregation of enzyme dodecamers along the 6-fold axes of symmetry.

(2) Bovine brain glutamine synthetase has 2 essential divalent cation sites/subunit -- a structural site and a higher affinity nucleotide-metal ion site which can not be filled until the first site is occupied by Mn(II) or Mg(II). Chloride ions produce structural perturbations that may be physiologically important.

(3) Calorimetric measurements of active-site ligand binding to E. coli glutamine synthetase continue.

(4) Studies on the mercurial-promoted release of zinc from E. coli aspartate transcarbamoylase (ATCase) have been completed. Studies on the rebinding of zinc ions to isolated regulatory subunits of this enzyme are in progress. The results relate to changes in catalytic and regulatory chain interactions in ATCase molecules responsible for the allosteric properties of this enzyme and to mechanisms of dissociation and assembly of this enzyme.

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Project Description:

Objectives: (1) In general, to study conformation and stabilization changes of biologically important protein macromolecules promoted by the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation. To study protein-metal ion interactions by kinetic and equilibrium methods to determine structural and catalytic roles of specific divalent cations. Spectrophotometric, fluorometric, ultracentrifugal, calorimetric, electrophoretic, chromatographic, enzyme kinetics, stopped-flow kinetics, and collaborative electron microscopic techniques are applied as required.

(2) To study the physical and chemical properties of unadenylylated and adenylylated glutamine synthetases (GS) from Escherichia coli, particularly with respect to correlating the regulation, structure and catalytic function of GS. The unusual properties of the complex formed when ADP, L-methionine-S-sulfoximine phosphate, and two divalent cations are bound to active sites of E. coli GS are being used to investigate the effects of these tightly bound active-site ligands on the tertiary and quaternary structure of the dodecameric enzyme and to obtain estimates of inter-subunit and intra-subunit distances by introducing nucleotide analogs as structural probes at active sites and at adenylylation sites of the enzyme. Covalent modification of GS with other nucleotide analogs will locate this interaction site in the subunit primary structure.

(3) To study active-site ligand interactions with mammalian GS in order to detect any structural homology with E. coli GS. For this purpose, GS from bovine and ovine brain are being purified and characterized.

(4) To study Zn^{2+} bonding domains of aspartate transcarbamoylase (ATCase) from E. coli and of isolated regulatory subunits of ATCase in order to better understand the structural role of Zn^{2+} in the intact enzyme and in the assembly of ATCase from regulatory and catalytic subunits.

Major Findings:

(1) The use of nucleotide analogs as structural probes of E. coli glutamine synthetase.

E. coli glutamine synthetase (GS) is composed of 12 identical subunits (M_r 50,000) arranged in 2 superimposed hexagonal rings of ~ 140 Å in diameter. Each subunit has an active site which binds substrates and 2 divalent metal ions and a regulatory site near the active-site which contains a tyrosyl residue that can be covalently modified with AMP. Centers of subunits from either ring are ~ 45 Å apart.

As summarized in the annual report of 1983-84, M.R. Maurizi has prepared various derivatives of ATP substituted at the 8- or 6-position of the adenine ring. These were shown to bind fairly tightly to GS ($K'_A > 10^5$ M^{-1}) and to substitute for ATP in the auto-inactivation reaction of Mn•GS with L-met-S-sulfoximine (MSOX) at pH ~ 7 (J. Biol. Chem. 257, 4271-4278, 1982). With MSOX phosphate, 2 Mn^{2+}, and the corresponding analog of ADP tightly bound at a subunit active site ($K'_A > 10^{12}$ M^{-1}) both intra- and inter-subunit contacts are strengthened (J. Biol. Chem. 257, 4271, 7246, 1982).

Recently, we found also that the inactive enzyme comples has a substantially more compact structure than has the native enzyme [$(\Delta\underline{s}/\Delta\underline{s})_{corr}$ = + 1.1% in sedimentation velocity studies]. By forming the inactive complex with various derivatives of ATP substituted in the 6- or 8-position, specific probes have been introduced into the active sites of GS for spectrophotometric, fluorometric, electron microscopic, and X-ray crystallographic studies. In addition, some of these ATP analogs can be introduced into adenylylation sites by adenylyl-transferase-catalyzed adenylylation to act as an additional structural probe of GS. Thus, specific, well defined sites of GS can be labeled with structural probes without interference from nonspecific labeling.

(a) Fluorescence energy transfer distances between nucleotide probes at active sites of glutamine synthetase (Investigators: M.R. Maurizi, P.G. Kasprzyk, and A. Ginsburg).

The distance between active sites of dodecameric GS was measured by fluorescence energy transfer taking advantage of the essentially irreversible binding of ATP analogs in the presence of MSOX and Mn^{2+}. Two fluorescent ATP derivatives were used: the first was obtained by alkylation of 8-mercapto ATP with N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (IAEDANS) to produce AEDANS-ATP; the second was 1-N^6-etheno-2-aza ATP (aza-ε-ATP). The acceptors were obtained by alkylation of 6-mercapto purine ribonucleoside triphosphate or 8-mercapto ATP with 4(p-dimethylaminophenylazo)phenyl-4-iodoacetamide to produce 6Y-ATP or 8Y-ATP, respectively. The procedure for the energy transfer experiments was first to obtain GS with an average of 1 and 2 fluorescent ligands per dodecamer by partially inactivating the enzyme with MSOX, Mn^{2+}, and limiting amounts of either AEDANS-ATP or aza-ε-ATP. The remaining active sites were then inactivated with MSOX, Mn^{2+}, and either 6-Y-ATP or 8-Y-ATP as acceptor or with underderivatized ATP instead of acceptor for controls. Inactive protein derivatives were then gel filtered through P-10 columns in buffer at pH 7.0 to remove free and loosely bound ligands. The difference in fluorescence yield between the fully inactive complexes with ADP or with Y-ADP occupying most of the active sites was taken as a measure of fluorescence energy transfer. The efficiency of energy transfer (E) was then used to calculate the distance between pairs of active sites according to the Förster equation, $R = R_0(E^{-1}-1)^{1/6}$. Results of experiments performed with an average of 1 and 2 fluorescent donors per dodecamer were averaged and are summarized in Table I.

Table I. Distances between Active-site Probes on Glutamine Synthetase

Donor/Acceptor	R_0[a] (Å)	Transfer Efficiency %	R[b] (1 acceptor) (Å)	R[b] (2 acceptors) (Å)	R[b] (3 acceptors) (Å)
AEDANS-ADP/6Y-ADP	41	26 ± 4	50	57 ± 2	61
AEDANS-ADP/8Y-ADP	41	22 ± 4	52	59 ± 2	63
Aza-ε-ADP/6Y-ADP	42	32 ± 2	49	56 ± 1	60
Aza-ε-ADP/8Y-ADP	43	37 ± 4	47	55 ± 1	60

[a] R_0 is the critical transfer distance where the efficiency of energy transfer is 50%.

[b] R is the calculated distance between active-site probes on GS (with error estimates) assuming that transfer occurs to a single acceptor or to 2 or 3 equidistant acceptors on adjacent subunits.

The distances calculated using the four combinations of donor and acceptor agreed well (Table I) and indicated that the active sites on GS are widely separated. The low efficiency of energy transfer observed (together with the known geometry of the enzyme) suggests that fluorescence energy transfer is from a single donor to 2 or 3 acceptors on adjacent subunits. The calculated distance between equidistant active-site probes on heterologously bonded subunits within the same hexagonal ring is 56-61 Å. Probes on isologously bonded subunits are no closer than 60 Å and may be as far apart as 78 Å. Thus, active sites are away from the 6-fold axis of symmetry toward the outer edges of the dodecamer and are located ≥ 30 Å from the plane separating the hexagonal rings.

Active-site nucleotide probes on GS have allowed us to measure the kinetics of Zn^{2+}-induced face-to-face aggregation of enzyme dodecamers along the 6-fold axes of symmetry. The same fluorescent enzyme derivatives that were used for intramolecular energy transfer could be made to form linear face-to-face aggregates by the addition of low concentrations of $ZnCl_2$ in the presence of 9 mM $MgCl_2$ at pH 7.0. With nonspecific divalent cation sites occupied by Mg^{2+}, Zn^{2+} deforms the dodecamer by binding to a high-affinity site of each subunit that is distinct from active-site Mn^{2+} sites. Recall that fluorescent enzyme derivatives contained 1 or 2 eq per dodecamer of either of two fluorescent ADP analogs, 8-mercapto ADP alkylated with N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (AEDANS-ADP) or 1-N-^{6}etheno-2-aza-ADP (Aza-ε-ADP), with remaining active sites occupied by ADP or ADP modified with a fluorescence energy acceptor [4-(p-dimethylaminophenylazo)phenyl-4-thioacetamide attached to either the 6 or 8 position of the purine ring]. Zn^{2+}-induced stacking of these enzyme derivatives resulted in ~ 54-70% quench of fluorescence only when acceptors were present on the separate dodecamers. The fluorescence quench as a function of time correlated well with the degree of linear polymer formation. The time-dependent fluorescence quench during Zn^{2+}-induced face-to-face aggregation at pH 7.0 and 25°C had a second-order rate constant of ~ 10^5 s^{-1} M^{-1} at early stages, pseudo-first-order kinetics at later stages ($k \simeq 1.9\text{-}5.1 \times 10^{-3}$ s^{-1}) when the concentration of dodecamers was low and that of polymer ends about constant, and reached a maximum when the average n-mer was 6 dodecamers. With AEDANS-ADP, but not with aza-ε-ADP, at active sites, there was evidence of intramolecular isomerization during the stacking process that may relate to the flexibility of the fluorescent probe. An Arrhenius activation energy of 22.3 ± 0.2 kcal/mol was obtained for the face-to-face aggregation of dodecamers in the presence of 46 μM Zn^{2+} and 9 mM $MgCl_2$ at pH 7.0.

The kinetic studies conducted here should be generally applicable to studying self-assembly reactions of other proteins when donor and acceptor probes are attached. Also, the Zn^{2+}-induced stacking of GS dodecamers can be fully reversed by EDTA addition. Further studies on the reversibility of the Zn^{2+}-induced stacking reaction and on the kinetics of depolymerization are planned. If successful, this system can be used to test current theories on self-assembly and nucleation of two-dimensional polymers. Measurements of thermodynamic parameters also will aid in characterizing the types of intermolecular bonding involved in face-to-face aggregation of GS.

In order to estimate the intermolecular distance between active-site probes on layered dodecamers, it was necessary to correct the observed percent maximum quench values of 54-70% for unquenched polymer ends. Since the average n-mer was

6 dodecamers when the maximum quench was observed (from light scattering measurements of STEM images taken at 30 minutes by Dr. J.J. Lipka at the Brookhaven National Laboratory), corrections of 8% and 17% were added to observed percent quench values for 1 and 2 eq of fluorescent donor per dodecamer, respectively. When 8S AEDANS-ADP was the fluorescent probe, a correction of ~ 10% also was subtracted from the observed percent quench value for intramolecular isomerizations that occurred during the stacking process. After applying the above corrections to the observed percent quench values, the calculated intermolecular fluorescence energy transfer distances were in good agreement (± 1 Å with 6 of the 8 donor/acceptor pairs used) and indicated that the average separation between nucleotide probes on face-to-face aggregates is ~ 36 Å. This intermolecular energy transfer distance confirms that active-site nucleotide probes are toward exterior surfaces away from the lateral plane between hexagonal rings of the dodecamers.

(b) Labeling specific sites of glutamine synthetase with mercapto nucleotide•platinum (II) complexes from Salmonella typhimurium (Investigators: A. Ginsburg, M.B. Blackburn, and P.J. McFarland in collaboration with the laboratory of David Eisenberg at UCLA).

Fully unadenylylated GS from a mutant of S. typhimurium lacking adenylyltransferase is purified at UCLA and sent here. We have prepared coordination complexes between 8-mercapto ATP or 6-mercapto purine ribonucleoside triphosphate and aquo glycyl-L-methionato platinum (II) and subsequently bound these nucleotide analogs to all active sites of the enzyme by phosphorylation of MSOX in the presence of Mn^{2+}. Thus, we have specifically introduced an electron dense probe into each active site of the dodecamer (Pt(II) per subunit). These derivatives were shipped to UCLA for X-ray crystallographic studies. Unfortunately, the 8S-ADP•Pt(II) enzyme complex crystallized in a different form than that already analyzed at ~ 4 Å resolution. Growth of crystals of the 6-S-ADP•Pt(II) complex is in progress.

We have just succeeded in fully adenylylating the enzyme from S. typhimurium with 6-mercapto purine ribonucleoside triphosphate. Because the 6S-AMP group at adenylylation sites was found to be extremely light sensitive, the coordination complex with aquo glycyl-L-methionato•Pt(II) was made as quickly as possible. The Pt(II) complex was stable to light and, furthermore, was not disrupted by the addition of mercurial or thiol reagents. The enzyme derivative containing 6S-AMP•Pt(II) complex at adenylylation sites, repurified by gel filtration, had an A_{278nm}/A_{320nm} ratio of ~ 2.6 and was ~ 80% active at pH 7.57. This derivative will be further characterized and then shipped to UCLA for crystallization. X-ray analysis of this enzyme derivative should yield valuable 3-dimensional structural information on the locations of adenylylation sites.

(c) Location of 1 or 2 tryptophanyl residues near the active site of the GS subunit (Investigators: P.J. McFarland and A. Ginsburg in collaboration with Dr. Jay R. Knutson, Laboratory of Technical Development, NHLBI).

The 2.5-fold enhancement of tryptophanyl residue fluorescence produced by ATP binding is 100% or 67% quenched when 6S-ATP or 8S-ATP, respectively, is bound at active sites (M.R. Maurizi and A. Ginsburg). The addition of a mercurial reagent decreases the overlap between tryptophanyl residue fluorescence and

absorbance of the mercaptonucleotides and partially relieves the quench. Fluorescence lifetime studies with a laser light source by J. Knutson of unadenylylated and adenylylated native Mn•GS and inactive enzyme complexes containing Mn^{2+}, MSOXP, and ADP, 6S-ADP, or 8S-ADP at active sites are in progress. In addition, corrected fluorescence emission spectra at magic angle (polarizer in the vertical position and emission at 55° from the vertical) are being collected. The results of Jay Knutson show that there are 3 exponentials in lifetime measurements (0.4-6ns) and that the relative intensities shift with derivatization of the enzyme. The complexities are still being sorted out but it appears that ~ 50% of the effects observed by substitution of 6S-ADP or 8S-ADP for ADP at active sites is due to energy transfer to 1 or 2 tryptophanyl residues while 50-60% of the intensity changes are decoupled from energy transfer (i.e., due to a conformational change). These studies will eventually give information on intra-subunit interactions.

(2) Thermodynamics of active-site ligand binding to glutamine synthetase from E. coli (Investigators: M.B. Blackburn and A. Ginsburg).

Active-site ligand interactions with dodecameric GS from E. coli continue to be studied by calorimetry. The LKB batch-type microcalorimeter used in previous work has been modified. The installation of a microtitration device now allows multiple serial additions of ligand to protein, which facilitates the construction of binding curves. The calorimeter has also been interfaced to a computer to allow unattended operation, to improve the accuracy and precision of signal integration, and to simplify data manipulation and storage. Software was written to acquire and store the data, to display it graphically, and to produce experimental documentation. The old integration system (ball and disc integrator/recorder) was used to demonstrate the redundancy of the new system (Simpson's rule summation of the digitized signal). A series of standard dilution and neutralization experiments was performed to establish the accuracy (relative error < 5%) and precision (rsd < 5%) of the computer-controlled microtitrator system.

After thoroughly testing the calorimeter in the batch and titration modes, binding experiments were resumed. Measurements were made with the unadenylylated enzyme at pH 7.1 in 20 mM Hepes/KOH, 100 mM KCl, and 1.0 mM $MnCl_2$ at 25 and 30°C. With the enzyme saturated with the ATP analog AMP-PNP, the binding of L-met-S-sulfoximine is stoichiometric ($K_A \approx 3 \times 10^5\ M^{-1}$) and substoichiometric additions of the latter ligand to ~ 25 mg of the Mn•GS-AMP-PNP complex gave linear heats until most all of the available active sites had been titrated. The average binding enthalpies at 25 and 30°C were -10.1 and -10.8 kcal (mol GS subunit)$^{-1}$ with a 5% rsd, giving an approximate value for ΔCp of 140 ± 100 cal/(°K•mol). With the Gibbs free energy of binding L-met-S-sulfoximine to the Mn•GS•AMP-PNP complex at 303°K equal to -7.6 kcal/mol under these conditions (measured by equilibrium binding), ΔS = -11 cal/deg•mol.

In order to compare thermodynamic parameters previously measured for substrates and substrate analogs binding to GS to those obtained by binding the transition state complex L-met-S-sulfoximine phosphate (MSOXP), the latter was repurified from frozen stock solutions [(previously prepared by a summer student, Stephen Pease, by phosphorylation of MSOX with ATP using GS catalysis and recycling of the enzyme under reactivation conditions (M.R. Maurizi and A. Ginsburg,

J. Biol. Chem. 257, 4271-4278, 1982)]. The compound MSOXP was reisolated from breakdown products by paper chromatography (ascending in ethanol:ammonium acetate, 70:30) and elution of the separated compound with water. The purity and concentration of MSOX solutions were determined by titrations of Mn•GS with this ligand in the absence and presence of ATP and measuring "inactivation" units under the two conditions. (In the absence of ATP, MSOXP inactivates GS, whereas ATP is required for MSOX to inactivate the enzyme.) Then, the enthalpy of binding L-met-S-sulfoximine phosphate to Mn•GS was measured at pH 7.1 and 30°C; $\Delta H = -13.2 \pm 0.3$ kcal(mol subunit)$^{-1}$. If $K \approx 10^{16}$ M^{-1} for the extremely tight binding of this inactivating ligand at pH 7.1, $\Delta G' \simeq -22$ kcal/mol and $\Delta S + 29$ cal/deg•mol. Thus, the tight binding of L-met-S-sulfoximine to the Mn•GS•AMP-PNP complex is enthalpically controlled at pH 7.1 and 30°C, whereas the binding of the inactivating ligand L-met-S-sulfoximine phosphate to Mn•GS under the same conditions is both enthalpically and entropically controlled.

(3) Studies on the binding of active-site ligands and metal ions to glutamine synthetase from bovine brain (Investigators: H.B. Pinkofsky in collaboration with M.R. Maurizi).

The native, catalytically active form of mammalian GS is an octomer. Despite extensive studies on this enzyme, the roles of divalent cations and activators in catalysis were poorly understood.

The purified bovine brain GS (400 U/mg in the Mn^{2+}-supported glutamyl transfer assay at pH 6.8 and at least 95% pure in SDS-slab gel electrophoresis) has a specific absorption coefficient at 280 nm of 1.50 ± 0.02 which alters published stoichiometries of Mn^{2+} binding. We also have found that the enzyme is stable for several months at 4°C when stored in the presence of 50 mM K-PO_4 (pH 6.8) and 1 mM EDTA in the absence of Cl^- and of metal ions, which makes many studies now possible.

To explore the catalytic requirements, saturation functions for Mn^{2+}, Mg^{2+}, and ADP were obtained in the γ-glutamyl transfer reaction at pH 7.2. Saturation plots for Mn^{2+} ions were sigmoidal; $[S]_{0.5}$ values were 1 μM and 2 mM with Hill coefficients of 2.1 and 1.2 for Mn^{2+} and Mg^{2+}, respectively. Values of K_m were 19 nM for ADP-Mn and 7.2 μM for ADP-Mg. Furthermore, a Job analysis of the ADP- and Mn^{2+}-supported γ-glutamyl transfer activity showed that all subunits of the bovine brain GS express activity at 1 eq of ADP bound/subunit. Inactivation of the enzyme with MSOX and ATP in the presence of Mn^{2+} or Mg^{2+} resulted in the tight binding of 2 eq of Mn^{2+} or Mg^{2+} and 1 eq each of ADP and MSOXP per subunit. These data indicate that GS from bovine brain has two essential M^{2+} sites per subunit for Mn^{2+} or Mg^{2+} that must be saturated for activity expression -- as has been found for E. coli GS.

Mn^{2+} or Mg^{2+} binding to GS from bovine brain produces conformational changes. Mn^{2+} ($K_D \simeq 4.2$ μM) or Mg^{2+} ($K_D \simeq 1.4$ mM) quenches tryptophanyl residue fluorescence; Cl^- produces an additional quench ($K_D' \simeq 120$ μM). This latter effect appears to be specific for the Cl^- ion since other anions such as acetate, nitrate, or borate at < 1 mM concentration did not have this effect. Both Mn^{2+} and Mg^{2+} also produce protein UV difference spectra with peak-troughs at 292-299, 292-288, and 280-288 nm. Spectrophotometric titrations with Mn^{2+} and Mg^{2+} gave

K_D' values of 10.2 μM and 1.8 mM, respectively. Chloride ion ($K_D' \simeq 0.13$ mM) also produced a spectral perturbation. Interestingly, the magnitudes of the UV spectral perturbations and of the quench of tryptophanyl residue fluorescence produced by binding of 1 eq/subunit of Mn^{2+} or Mg^{2+} were increased by the presence of saturating chloride ion, although K_D' values for M^{2+} were unchanged.

Equilibrium binding of Mn^{2+} to bovine brain GS at pH 7.0 in the absence and presence of ADP was measured by atomic absorption -- after ascertaining that the purified enzyme contained no bound Mn^{2+}. The Mn^{2+} binding data could be fit by a sequential model of Mn^{2+} binding to each subunit in which the ADP-Mn complex can be bound ($K_A' \simeq 10^6\ M^{-1}$) only after the first site for Mn^{2+} is occupied ($K_A' \simeq 1.5 \times 10^5\ M^{-1}$). Measurements of [^{3}H]ADP binding to the Mn•enzyme gave $K_A' \simeq 1.1 \times 10^6\ M^{-1}$ with a stoichiometry of binding equal to ~ 1 eq of ADP-Mn/subunit. [Also, binding of [^{3}H]ADP in the presence of 10 mM phosphate (which increases the affinity of the enzyme for ADP-Mn ~ 53-fold) gave a stoichiometry of binding equal to 1.0 ADP-Mn/subunit.] The agreement between the K_A' value for ADP-Mn binding in the absence of P_i or arsenate and that deduced from Mn^{2+} binding measurements in the presence of ADP suggests that the sequential model is correct. The fact that the nucleotide-Mn complex has a higher affinity at a second site than has the structural site for Mn^{2+} (which must be filled first) explains the sigmoidal curves of activity expression vs $[Mn^{2+}]_{free}$, since both sites must be occupied for activity expression. Affinity-labeling of nucleotide sites are being attempted.

It has been claimed by F.C. Wedler that mammalian brain GS are Mn-enzymes. We have found no evidence that this is the case. However, we are purifying the GS from ovine brain from which Wedler draws this conclusion to see if this enzyme differs from the bovine brain enzyme. Also, we plan to examine the metal ion content of GS in homogenates of brain using a rabbit antibody directed against the bovine brain enzyme to precipitate GS from crude extracts after fixing in vivo bound M^{2+} to GS by addition of MSOXP. If successful, this should show whether or not most of the Mn^{2+} in the brain is sequestered by GS.

(4) Aspartate transcarbamoylase from E. coli: Studies on the Zn^{2+} bonding domains (Investigators: J.B. Hunt (The Catholic University of America, Washington, D.C.), S.H. Neece, and A. Ginsburg in collaboration with H.K. Schachman at the University of California, Berkeley).

Aspartate transcarbamoylase (ATCase) from E. coli contains 6 catalytic (c) chains and 6 regulatory (r) chains (c_6r_6); the 4 -SH groups of each r chain are involved in tetrahedral bonding of Zn^{2+} near the c:r contact region (Monaco et al.: Proc. Nat. Acad. Sci. U.S.A. 75, 5276, 1978). Mercurials dissociate ATCase (Gerhart and Schachman: Biochemistry 4, 1054, 1965). The release of Zn^{2+} from ATCase and from isolated r subunit, upon challenge by p-hydroxymercuriphenylsulfonic acid (PMPS), and the rebinding of Zn^{2+} by these proteins, upon displacement of PMPS w h 2-mercaptoethanol, have been studied using the sensitive high-affinity metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) at pH 7.

Studies on the mercurial-promoted release of Zn^{2+} from ATCase have been completed and were summarized in last year's Annual Report.

Studies of Zn^{2+} release and rebinding to isolated regulatory dimers are in progress. The fact that EDTA removes Zn^{2+} from regulatory dimers, whereas PAR does not, sets lower and upper limits for the affinity constant of regulatory dimers for Zn^{2+}; i.e., $10^{12} \leq K_A' \geq 10^{14}$ M^{-1}. Mercurial-promoted Zn^{2+} release is > 3000-fold faster from r_2 subunits than from ATCase. The rebinding of Zn^{2+} from PAR was rate-limiting. Also, the PAR-Zn^{2+} complex was found to deliver Zn^{2+} to r_2 subunits. Experiments are underway to separate these kinetic steps in the binding of Zn^{2+} to regulatory subunits.

Significance to Biomedical Research: The regulation and control of enzymic activities *in vivo* is of fundamental importance in cellular metabolism. *In vitro* studies of conformation and stabilization changes of biologically important protein macromolecules promoted by the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation are important in understanding cellular processes on a molecular level.

Proposed Course:

(1) To study conformational and stabilization changes of protein macromolecules effected through the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation. Ultracentrifugation, calorimetry, spectral, viscometry, fluorescence, equilibrium binding, pH, electrophoretic, and kinetic techniques will be used.

(2) To study mutual interactions of divalent cations, substrate (or substrate analogs), and inhibitors with glutamine synthetase from *E. coli*. To locate specific sites in the subunit and the dodecameric structures in order to better understand tertiary and quaternary structural changes that occur on ligand interactions with this enzyme.

(3) To characterize the reversible thermal transition of *E. coli* GS (discovered by A. Shrake while in this laboratory) more fully in terms of kinetic parameters and van't Hoff enthalpic changes.

(4) Studies on active-site ligand interactions with mammalian GS will continue. For this purpose, GS from bovine and ovine brain have been purified. In addition, identification of divalent cations bound to those brain enzymes *in vivo* will be attempted.

(5) Studies on the removal of Zn^{2+} from isolated regulatory subunits of *E. coli* aspartate transcarbamoylase (ATCase) and on the rebinding of Zn^{2+} to regulato y subunits will continue in order to better understand the mechanism of assembly of ATCase from catalytic and regulatory chains.

Publications:

Pinkofsky, H. B., Ginsburg, A., Reardon, I., and Heinrikson, R. L.: Lysyl residue 47 is near the subunit ATP-binding site of glutamine synthetase from *Escherichia coli*. *J. Biol. Chem.* 259: 9616-9622, 1984.

Maurizi, M. R. and Ginsburg, A.: Active-site ligand binding and subunit interactions in glutamine synthetase from Escherichia coli. In Levine, R. L. and Ginsburg, A. (Eds.): Current Topics in Cellular Regulation: Modulation by Molecular Interactions, New York, Academic Press, Vol. 26, in press.

Hunt, J. B., Neece, S. H., Schachman, H. K., and Ginsburg, A.: Mercurial-promoted Zn^{2+} release from Escherichia coli aspartate transcarbamoylase. J. Biol. Chem. 259: 14793-14803, 1984.

Hunt, J. B., Neece, S. H., and Ginsburg, A.: The use of 4-(2-pyridylazo)-resorcinol in studies of zinc release from Escherichia coli aspartate transcarbamoylase. Anal. Biochem. 146: 150-157, 1985.

Maurizi, M. R. and Ginsburg, A.: ATP analogs as structural probes for Escherichia coli glutamine synthetase. Biochemistry, in press.

Maurizi, M. R., Kasprzyk, P. G., and Ginsburg, A.: Fluorescence energy transfer distances between active sites in glutamine synthetase from Escherichia coli. Biochemistry, in press.

Ginsburg, A., Kaspryzk, P. G., and Maurizi, M. R.: Intermolecular fluorescence energy transfer between active-site probes in stacked dodecamers of Escherichia coli glutamine synthetase. Biochemistry, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00205-30 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Occurrence and Biochemical Roles of Selenium in Selenoproteins and Seleno-tRNAs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Thressa C. Stadtman, Chief, Section on Intermediary Metabolism and Bioenergetics, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)* Gas Research Institute, Chicago, Illinois.
Dr. Raymond Burk, University of Texas Health Science Center, San Antonio, Texas.
Dr. August Böck, University of München, München, West Germany.
Dr. Harlan Wood, Case Western Reserve University, Cleveland, Ohio.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)* A clostridial glycine reductase complex catalyzes the reductive deamination of glycine to acetate and ammonia with the concomitant esterification of orthophosphate and synthesis of ATP. Use of hydrophobic chromatographic matrices such as phenyl and octyl sepharoses has greatly facilitated the separation of the two membrane-associated proteins (B and C) that are components of the glycine reductase complex. In the separation procedures used earlier, the C protein co-migrated with an iron protein. However, using Iron-55 labeled enzyme preparations it has been shown that the C protein does not contain iron. The C protein preparations currently studied are not inhibited by treatment with radical scavengers such as hydroxylamine or hydroxyurea. Thus, the postulated similarity between C protein and E. coli ribonucleotide reductase protein B does not appear to exist. The latter protein contains a tyrosine radical stabilized by ferrous iron which participates in the catalytic reaction. A collaborative project on purification and identification of a rapidly turning over selenocysteine-containing protein synthesized by liver and exported to the serum is in progress with Dr. Raymond Burk of the University of Texas, San Antonio. The biological mechanism of formation of selenocysteine residues in selenium-dependent enzymes is investigated in two different bacterial systems. The selenoprotein component of Clostridium sticklandii glycine reductase has been partially sequenced around the single selenocysteine residue. Synthetic RNA probes corresponding to this amino acid sequence then can be used to isolate the complementary gene sequence which should specify the nature of the amino acid precursor of the selenocysteine residue. A collaborative project with Dr. August Böck of München has a similar objective. Using his cloned gene from E. coli that specifies a selenocysteine-containing subunit of formate dehydrogenase we will provide the requisite amino acid sequence for comparison with the DNA sequence (Böck's part of the project). Studies on the mechanism of methane production from acetate (Gas Research Institute-supported project) have started with isolation and study of carbon monoxide dehydrogenase (a nickel enzyme presumed to be involved).

Project Description:

Objectives:

1. Roles of trace elements (e.g., selenium, molybdenum, cobalt, and nickel), quinones, deazaflavins, and non-heme iron proteins in anaerobic electron transport processes.

(a) Purification and characterization of the protein C component of clostridial glycine reductase. Studies on the mechanism of glycine reduction and the coupled phosphate esterification reaction using enzyme complex reconstituted from purified protein components A, B, and C. Role of the selenoprotein component (protein A) and characterization of the phosphate ester that reacts with ADP to form ATP.

(b) Characterization of Methanococcus vannielii enzymes involved in methane biosynthesis: (1) A hydrogenase that contains selenocysteine, Ni, FeS centers, and FAD; and (2) a carbon monoxide dehydrogenase that contains Ni and FeS centers.

2. Biological mechanisms of selenium incorporation into selenoenzymes and seleno-tRNAs.

(a) Comparison of the amino acid sequences of selenocysteine-containing peptides with nucleotide sequences of corresponding DNA as a means of identifying the amino acid precursor of selenocysteine.

(b) Identification of selenium donor and mechanism of biosynthesis of 2-selenouridine residues in bacterial tRNAs.

3. Biochemical roles of bacterial and mammalian seleno-tRNAs. The strictly anaerobic bacteria contain the highest amounts of seleno-tRNAs suggesting a regulatory role in anaerobic processes.

Major Findings:

1. (a) The project to obtain sufficient quantities of the three protein components of clostridial glycine reductase for detailed reaction mechanism studies has been continued. Procedures for obtaining homogeneous preparations of protein A, a selenoprotein, and protein B, a carbonyl group protein, were developed previously. Using a series of chromatographic steps suitable for large scale work, highly-purified stable preparations of protein C have now been obtained. The hydrophobic chromatography matrices effectively separate proteins B and C and allow both to be recovered simultaneously after initial separation of protein A from crude extracts by ion exchange chromatography. ^{55}Fe-labeled cell extracts are used in some experiments to monitor the iron contents of protein fractions. Adenylate kinase activity, which contaminates protein C fractions after many purification steps, was not inhibited by di-adenosine pentaphosphate and did not bind to agarose-diadenosine pentaphosphate whereas the mammalian adenylate kinase is effectively removed by such a procedure. The ADP analog, β,γ-methylene ADP, which cannot be used by

adenylate kinase as substrate, also failed to substitute for ADP as phosphate acceptor in the glycine reductase reaction. Although adenylate kinase removal is essential for some of the planned mechanism studies, the ability of thiophosphate to substitute for orthophosphate circumvents some of these problems.

(b) A research project supported by a special grant from the Gas Research Institute of Chicago is currently carried out by Dr. David Grahame, a guest worker. This involves purification and characterization of enzymes from methane-producing bacteria that are involved in the conversion of acetate and various one-carbon compounds to methane. These catalysts participate in strictly anaerobic electron transport processes and contain a number of trace elements, e.g., Se, Ni, Mo, and Co, of special importance to the overall biochemical and nutritional projects of the laboratory. A carbon monoxide dehydrogenase which is implicated in reactions involving the biosynthesis and degradation of acetate is currently being studied.

2. (a) To test the possibility that the unusual amino acid, selenocysteine, occurs in selenoenzymes as the result of a posttranslational modification we have determined the amino acid sequences of selenocysteine-containing peptides isolated from selenoprotein A of the glycine reductase complex. The ultimate aim is to obtain the corresponding RNA and DNA so that the latter can be sequenced and from the sequence the identity of the precursor amino acid elucidated. This work is carried out primarily by Dr. Mark Sliwkowksi (see his individual project report). A collaborative effort with Dr. August Böck of the University of München, München, West Germany, is in progress to identify the DNA triplet that codes for the amino acid precursor of selenocysteine in an Escherichia coli formate dehydrogenase selenopeptide. The DNA that codes for this selenopeptide was cloned into a plasmid carrying a chloramphenicol resistance marker and is being sequenced by Böck. After growing the plasmid carrying E. coli mutant on ^{75}Se we have isolated the radioactive selenopeptide and will prepare a ^{75}Se-selenocysteine-containing peptide fragment for amino acid sequence analysis. Alignment of this sequence with the DNA sequence should then provide the desired information.

(b) The major selenonucleoside present in the seleno-tRNAs of three different bacterial species was identified as 5-methylaminomethyl-2-selenouridine (mnm^5-Se^2U). This selenonucleoside is present in the anticodon of the major glutamate accepting tRNA species of Clostridium sticklandii. In E. coli the corresponding thionucleoside (mnm^5-S^2U) occurs in the major glutamate and lysine accepting tRNA species whereas minor isoaccepting $tRNA^{Glu}$ and $tRNA^{Lys}$ species contain the selenonucleoside. Preliminary experiments on the biosynthesis of the selenonucleoside indicate that in E. coli the preformed thionucleoside in the tRNA is converted to the selenonucleoside. In the in vitro enzyme system ATP was required and selenite served as source of selenium. The presumed activation of S to make it a leaving group to be replaced by selenium from an as yet unknown donor is suggested. The mechanism whereby the levels of thio and seleno-tRNA isoacceptors are maintained in a fairly constant ratio is of special interest.

3. Amino acid transfer ribonucleic acids (tRNAs) are known to contain a variety of modified bases which are formed after synthesis of the polynucleotide structure. A few of these modified bases have been shown to exert regulatory functions and similar roles are to be expected in the case of the selenium modified tRNAs. The most strictly anaerobic microorganism we have studied, the methane-producing Methanococcus vannielii, has the highest content of seleno-tRNAs (15-20% of the total population). C. sticklandii seleno-tRNAs account for 5-8% of the total tRNA population whereas the facultative anaerobe, E. coli, has a lower content (about 4%) and none could be detected in a strict aerobe, Bacillus subtilis. Whether this apparent correlation is indicative of special roles of Se-tRNAs in highly anaerobic systems or is purely coincidental is presently unknown. The detection of a higher content of seleno-tRNAs in a mouse leukemia cell line as compared to some normal mouse tissues also is suggestive. Experiments of W.-M. Ching and A. Wittwer demonstrated that in an in vitro protein synthesizing system containing rabbit globin message, incorporation of radioactive glutamate and lysine into protein was 40% greater when the amino acids in ester linkage to seleno-tRNAs rather than to thio-tRNAs were added. This can be explained by the greater abundance of lysine and glutamate codons ending in G rather than A in the globin message and the relative preference of the seleno-tRNAs as compared to the thio-tRNAs for the codons ending in G. Such a preference points out the advantage of use of seleno-tRNAs for effective translation of certain cloned eukaryotic messages.

Proposed Course:

Continuation of the project on the precise role of the glycine reductase selenoprotein, particularly as regards the synthesis of ATP concomitant with glycine reduction to acetate.

Synthesis of an RNA probe complimentary to the amino acid sequence of the selenopeptide derived from the glycine reductase selenoprotein will be attempted. This is needed to locate the DNA that codes for the protein and eventual sequence of the DNA to determine the selenocysteine precursor.

A cooperative research project with Dr. August Böck of München, West Germany, should provide information concerning the origin of the selenocysteine residue in a formate dehydrogenase of E. coli.

A collaborative project with Dr. Harlan Wood of Case Western Reserve University will be undertaken to see if the carbon monoxide dehydrogenase of methane-producing bacteria resembles a carbon monoxide dehydrogenase involved in acetate biosynthesis. That is, does it catalyze an exchange reaction between carbon monoxide and the carboxyl group of acetyl-CoA? The latter reaction is catalyzed by the clostridial enzyme that also synthesizes acetate from a methyl group donor, carbon monoxide, and CoA-SH. It is suspected that the conversion of acetate to methane and CO_2 by methane bacteria shares some common mechanisms with the biosynthetic process.

Continuation of the joint effort with Raymond Burk to purify the selenoprotein from rat serum that is produced in the liver is planned when a student of Burk's arrives at Johns Hopkins University later in the year.

Honors:

Biochemical Representative on Ad Hoc Panel from U.S. to joint meeting of U.S. Board on Chemical Sciences and Technology and British Science and Engineering Research Council, Abingdon, England, December 16-18, 1984.

Invited speaker at 9th Enzyme Mechanisms Conference, Tarpon Springs, Florida, January 3-5, 1985.

New methane-producing organism named in honor of Thressa C. Stadtman: Methanosphaera stadtmaniae, gen. Nov., sp. nov.

Publications:

Ching, W.-M., Wittwer, A.J., Tsai, L. and Stadtman, T.C.: Distribution of two selenonucleosides among the selenium-containing tRNAs from Methanococcus vannielii. Proc. Natl. Acad. Sci. U.S.A. 81: 57-60, 1984.

Wittwer, A.J., Tsai, L., Ching, W.-M. and Stadtman, T.C.: Identification and synthesis of a naturally occurring selenonucleoside in bacterial tRNAs: 5-Methyl-amino-methyl-2-selenouridine. Biochemistry 23: 4650-4655, 1984.

Sliwkowski, M.X. and Stadtman, T.C.: Incorporation and distribution of selenium into thiolase from Clostridium kluyveri. J. Biol. Chem. 260: 3140-3144, 1985.

Ching, W.-M., Alzner-DeWeerd, B., and Stadtman, T.C.: A selenium-containing nucleoside at the first position of the anticodon in seleno-tRNA^{Glu} from Clostridium sticklandii. Proc. Natl. Acad. Sci. U.S.A. 82: 347-350, 1985.

Yamazaki, S., Tsai, L., Stadtman, T.C., Tashima, T., Nakaji, A., and Shiba, T.: Stereochemical studies of a selenium-containing hydrogenase from Methanococcus vannielii: Determination of the absolute configuration of C-5-chirally labeled dihydro-8-hydroxy-5-deazaflavin cofactor. Proc. Natl. Acad. Sci. U.S.A. 82: 1364-1366, 1985.

Davis, J.N. and Stadtman, T.C.: Purification and properties of a quinone-dependent p-nitrophenylphosphatase from Clostridium sticklandii. Arch. Biochem. Biophys. (Horecker Symposium Issue) 239: 523-530, 1985.

Stadtman, T.C.: Bacterial selenoenzymes and seleno-tRNAs. In Selenium in Biology and Medicine, 3rd International Symposium, Beijing, P.R.C., May 28-June 2, 1984. In press.

Stadtman, T.C.: Specific occurrence of selenium in certain enzymes and amino acid transfer ribonucleic acids. In Phosphorus and Sulfur, 11th International Symposium on Organic Chemistry of Sulfur, September 10-15, 1984. Lindau, Germany. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00206-26 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Stereochemical Studies of Enzymatic Reactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Lin Tsai, Research Chemist, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

Project 1: Professor Tetsuo Shiba, Osaka University, Japan
Dr. Shigeko Yamazaki, Merck, Sharp & Dohme,
West Point, Pennsylvania

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

(1) The absolute stereochemistry of the enzymic reactions involving 8-hydroxy-5-deazaflavin cofactor was established.

(2) Improved procedures for the syntheses of 5-substituted 2-selenouracil furnished good yields of 5-aminomethyl- and 5-carboethoxy-2-selenouracils.

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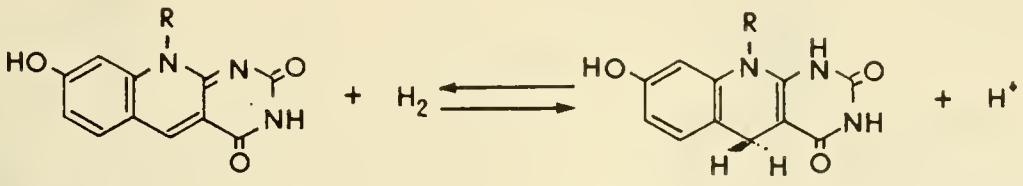
Project Description:

Objectives:

(1) The 8-hydroxy-5-deazaflavin cofactor present in Methanococcus vannielii and other methane-producing bacteria plays an important role as an electron carrier in the metabolism of these microorganisms. Although similar in some respect to the flavins, the 5-deazaflavin chromophore possesses the unique feature of having a diastereotopic, prochiral methylene group at C-5 of the reduced coenzyme. Thus, the redox system is amenable to stereochemical studies of the hydrogen transfer process.

A selenium-containing hydrogenase isolated from M. vannielii catalyzes the reduction of the 8-hydroxy-5-deazaflavin cofactor by molecular hydrogen:

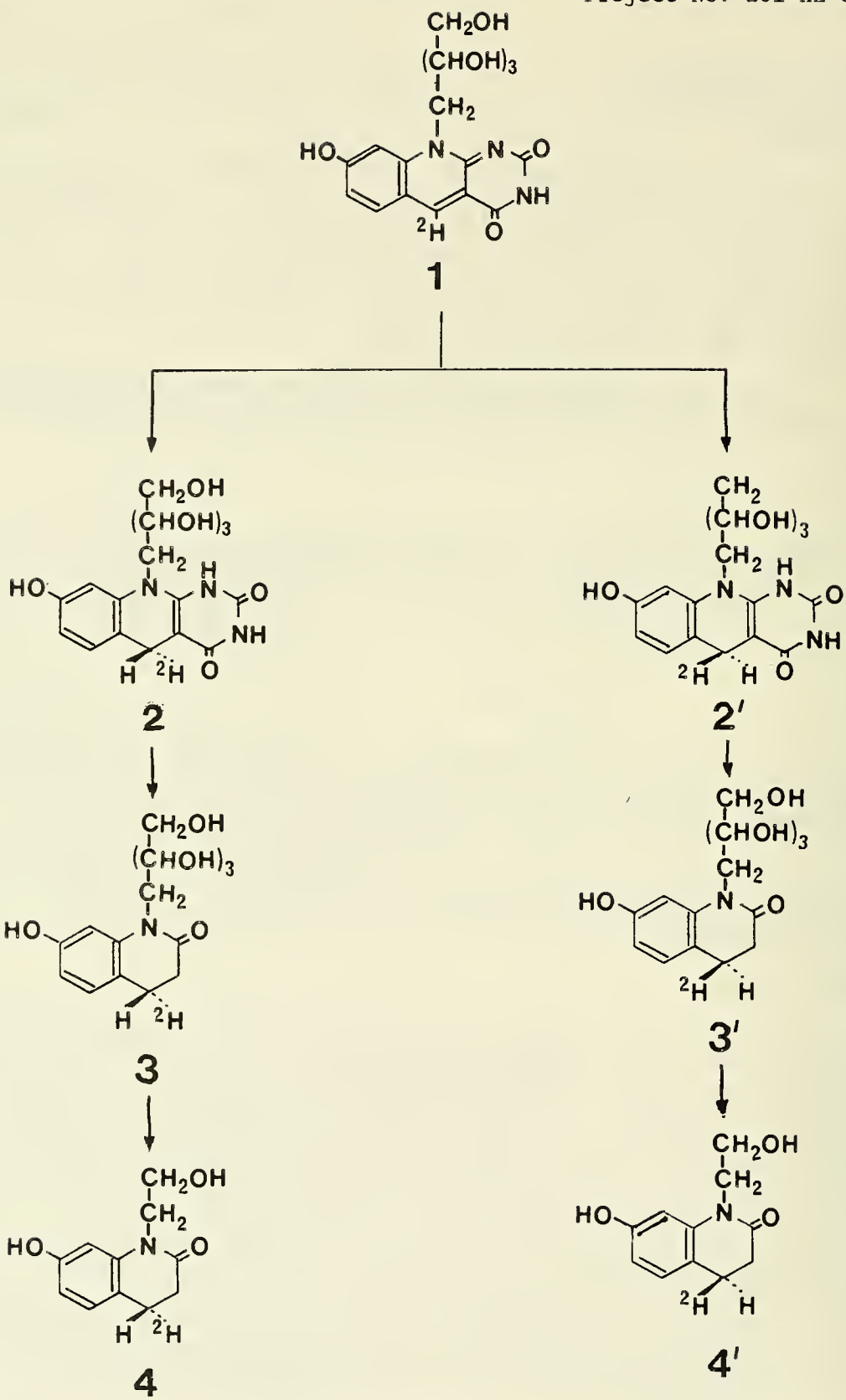


In order to study the stereochemistry of this reaction, it is necessary to examine the chirality at the C-5 center of the dihydro cofactor produced by the enzymic reaction on the 5-deutero-cofactor.

(2) In continuance of our study of selenium-modified nucleosides in tRNAs, it is important to develop methodology for the syntheses of various 5-substituted 2-selenouracils.

Major Findings:

(1) When the reduction catalyzed by the selenium-containing hydrogenase was performed with 7,8-didemethyl-8-hydroxy-[5-$^{2}H_{1}$]-5-deazariboflavin (1) as substrate, the dihydro cofactor could possess one of the two configurations, (2) or (2') (Scheme I). Since the dihydro compound could be readily oxidized upon exposure to air, the enzymic reduction product was immediately subjected to acid hydrolysis under anaerobic conditions to furnish the chemically stable deutero-lactam, (3) or (3'). This hydrolytic process involved only the cleavage of the pyrimidine ring of the molecule, the chiral integrity at the C-5 center was thus preserved. Hence, the absolute configuration of the deutero-lactam would be related directly to that of the dihydro-5-deazaflavin. The lactam, (3) or (3'), was further degraded to (4) or (4') so as to permit direct comparison with synthetic samples of known absolute configuration. Both enantiomers of the deutero-lactam, the S-form (4) and the R-form (4'), were synthesized from (R)- and (S)-3-hydroxy-3-phenylpropionic acid, respec-

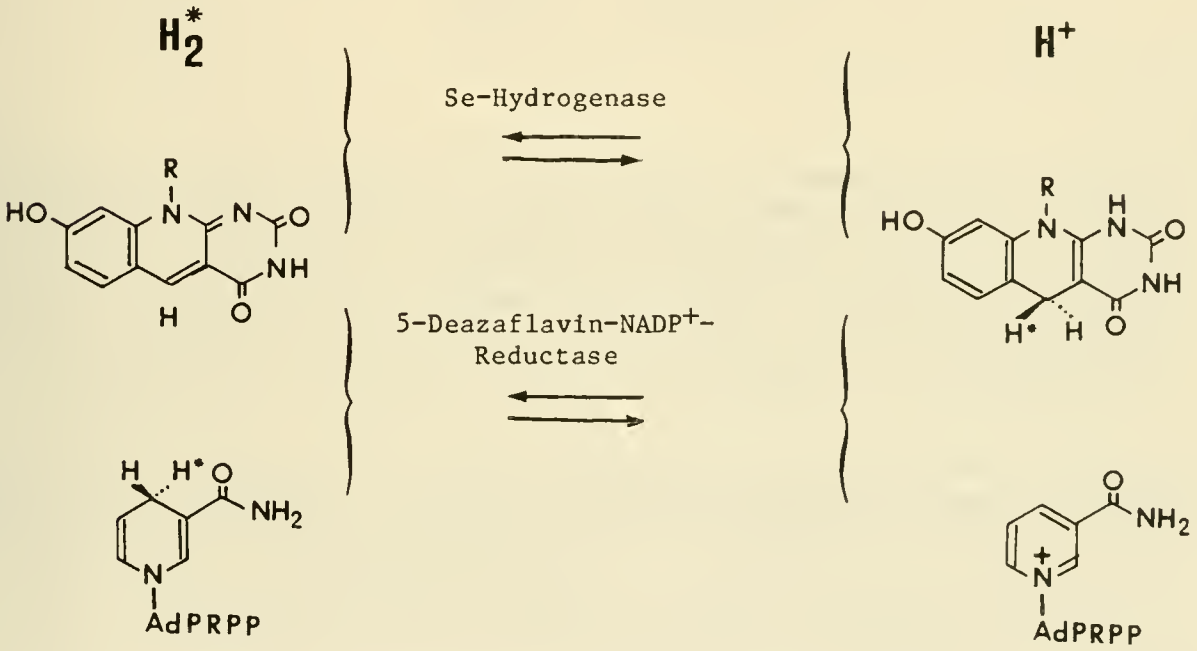


Scheme I

74

tively. Comparison of the ORD (optical rotatory dispersion) curve of the lactam from the enzymic reaction with those of the synthetic enantiomers, (4) and (4'), showed the enzymic product to be identical with the S-form (4). It follows, therefore, that the chiral label at the C-5 center of the dihydro-5-deazaflavin produced by the enzymic reaction must have the R-configuration as represented by (2) in Scheme I.

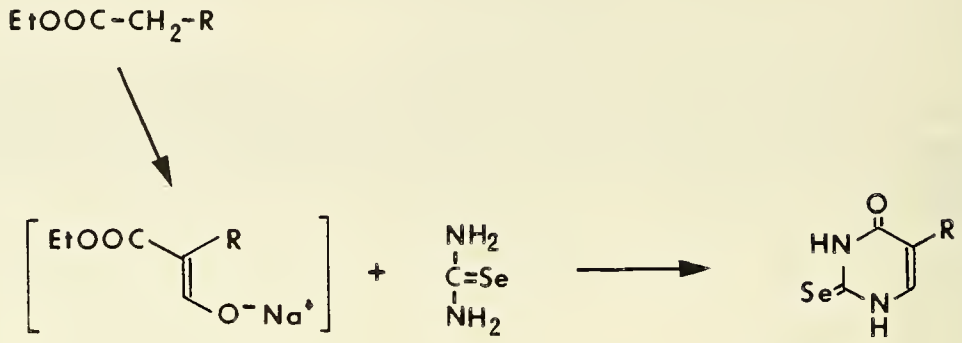
Since the relative stereochemistry of the 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase has previously been established and had also been shown to be the same as that of the selenium-containing hydrogenase, the absolute stereochemistry of both enzymic reactions can be expressed as in Scheme II.



Scheme II

The steric course of the enzymic reactions on the 8-hydroxy-5-deazaflavin cofactor can now be stated as follows: (a) The reduction of 8-hydroxy-5-deazaflavin cofactor occurs on the si face of the molecule, resulting in the introduction of a pro-S hydrogen at C-5; (b) the oxidation takes place by the abstraction of the pro-S hydrogen at C-5 of the dihydro cofactor.

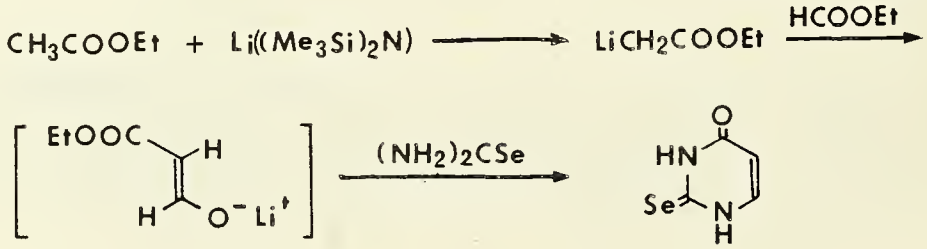
(2) We have demonstrated the feasibility of synthesizing 5-substituted 2-selenouracils by the condensation of selenourea with the enolate of β-aldehydo-esters generated in situ:



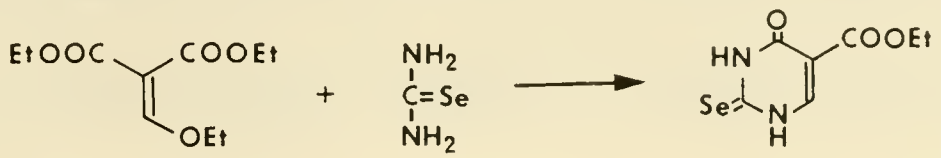
Although 5-methylaminomethyl-, ($R=CH_2NHCH_3$), and 5-aminomethyl-, ($R=CH_2NH_2$), 2-selenouracils have been synthesized by this method, it was difficult to control the reaction conditions to produce consistent results. The yield of 2-selenouracils varied from fair to very poor. The instability of selenium compounds could only be a part of the problem. After extensive experimentation, it was found that the production of the enolate of the β-aldehydo-ester was not consistent. Furthermore, the enolate, once formed, could undergo self-condensation and/or decomposition before reacting with selenourea.

One approach to overcome this problem was to use an excess of the starting ester. Thus, 5-formamidomethyl-2-selenouracil was obtained in 60-70% yield from selenourea by using 5-fold excess of ethyl 3-formamidopropionate. With the availability of material, it was possible to fully characterize this new 2-selenouracil by its UV, NMR, and mass spectroscopic properties. Hence, the identity of 5-aminomethyl-2-selenouracil with the acid degradation product of the selenonucleoside from the E. coli mutant was firmly established.

Another approach was to explore other means for the formation of the enolate. By using a more selective base, e.g., lithium bis(trimethylsilyl)-amide, than sodium hydride, ethyl formyl-acetate enolate was generated under very mild conditions. This procedure yielded 2-selenouracil in about 80%, a substantial improvement over the 30-50% yield obtained previously.



The use of an enol ether in place of the enolate provided yet another alternative. The highly activated enol ether, diethyl ethoxymethylenemalonate, reacted with selenourea at room temperature in thirty minutes to give 85% yield of a new seleno-compound, 5-carboethoxy-2-selenouracil.



Proposed Course:

To develop synthetic methods for various 2-selenouracils so as to help the identification of other selenium-modified nucleosides present in bacterial tRNAs.

Publications:

Teshima, T., Nakaji, A., Shiba, T., Tsai, L., and Yamazaki, S.: Elucidation of Stereospecificity of a Selenium-Containing Hydrogenase from Methanococcus vannielii. Syntheses of (R)- and (S)-[4-$^{2}H_{1}$]-3,4-dihydro-7-hydroxy-1-hydroxyethylquinolinone. Tetrahedron Letters 26: 351-354, 1985.

Yamazaki, S., Tsai, L., Stadtman, T.C., Teshima, T., Nakaji, A., and Shiba, T.: Stereochemical Studies of a Selenium-Containing Hydrogenase from Methanococcus vannielii: Determination of the Absolute Configuration of C-5 Chirally-Labeled Dihydro-8-hydroxy-5-deazaflavin cofactor. Proc. Natl. Acad. Sci. U.S.A 82: 1364-1366, 1985.

Wittwer, A., Tsai, L., Ching, W.-M., and Stadtman, T.C.: Identification and Synthesis of a Naturally Occurring Selenonucleoside in Bacterial tRNAs: 5-[(Methylamino)methyl]-2-selenouridine. Biochemistry 23: 4650-4655, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00211-12 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Protein Oxidation in Protein Turnover and in Aging

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB, NHLBI
Others:	A. Amici	Visiting Fellow	LB, NHLBI
	M. E. Wittenberger	Biological Laboratory Technician	LB, NHLBI
	B. S. Berlett	Biological Laboratory Technician	LB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	1.5	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It has been demonstrated that lysine and histidine residues in glutamine synthetase and glucose-6-phosphate dehydrogenase are among several amino acid residues that are oxidized to carbonyl derivatives by mixed-function oxidation systems. The Fenton reagent comprised of Fe-chelate and hydrogen peroxide was shown to catalyze the conversion of all common amino acids to ammonia and their corresponding α-keto acid derivatives. A number of physiological chelating agents (citrate, nucleoside di- and tri- phosphates, orthophosphate, pyrophosphate) as well as nonphysiological chelating agents (EDTA, EGTA, DETAPAC, o-phenanthroline, and α-dipyridyl) stimulate the oxidative deamination reaction about 2-4-fold. In addition to chelated iron, it was found that unchelated iron and also bicarbonate ion are essential for deamination to occur.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives: Previous studies in this laboratory have shown that mixed-function oxidation (MFO) systems catalyze the inactivation of enzymes. This inactivation is often accompanied by the loss of one histidine residue and by the formation of one or more carbonyl groups per subunit. The present study was designed to identify the amino acid residues that are precursors of the protein carbonyl groups generated by MFO catalyzed oxidation and to investigate the mechanism of amino acid oxidation by MFO systems.

Major Findings:

To facilitate identification of the amino acid residues of proteins that are oxidized to carbonyl derivatives by MFO systems, the carbonyl groups of oxidized glucose-6-phosphate dehydrogenase and glutamine synthetase were reduced to the corresponding hydroxy derivatives with sodium borotritide. Because the hydroxyl amino acids thus formed contained one nonexchangeable tritium atom they were readily identified as radioactive compounds following acid hydrolysis of the proteins and chromatographic separation of the free amino acids. For comparison, the amino acid digests of oxidized and subsequently tritiated poly-L-lysine and poly-L-histidine were also examined. Based on these studies it is deduced that lysine and histidine are among the five amino acid residues of enzymes that are modified by MFO systems. A nonenzymatic MFO system comprised of Fe^{2+}, hydrogen peroxide, and iron-chelating agents was found to catalyze the oxidative deamination of all the common amino acids. From detailed studies on the oxidation of leucine by this system, it was established: (a) that oxidative deamination requires the presence of both chelated and nonchelated iron, (b) that the oxidative deamination is greatly stimulated by bicarbonate ion and also by nucleotide di- and tri- phosphates, and (c) that O_2 is a by-product of the H_2O_2-mediated amino acid oxidation. The results are consistent with a mechanism in which hydroxyl radical and superoxide anion are intermediates.

Significance to Biomedical Research and Program of the Institute:

Results of these studies relate to the implication of mixed-function oxidation reaction in the regulation of protein turnover and in the accumulation of altered enzymes during cell aging. Fundamental to both processes is the generation of activated oxygen species (superoxide anion, hydroxyl radicals, etc.) which are thought to be involved in oxygen toxicity, and in mutagenesis responsible for development of lung cancer, especially in smokers, and in the excessive deposition of collagen.

Proposed Course:

Studies on the mechanism of amino acid oxidation by the Fenton reagent will be continued in an effort to identify the roles of bicarbonate ion, and both free and chelated iron in oxygen radical generation. Attempts will be made to obtain antibodies that recognize the oxidized amino acid residues in MFO-treated poly-L-lysine and poly-L-histidine. Such antibodies should be useful in the isolation and characterization of the altered (oxidized) proteins that occur during aging.

Publications:

Shacter, E., Chock, P. B., and Stadtman, E. R.: Regulation through phosphorylation/dephosphorylation cascade systems. J. Biol. Chem. 259: 12252-12259, 1984.

Federici, M., Chock, P. B., and Stadtman, E. R.: Interaction of Cibacron Blue F_3GA with glutamine synthetase: Use of the dye as a conformational probe. 1. Studies with unfractionated dye samples. Biochemistry 24: 647-660, 1985.

Federici, M. and Stadtman, E. R.: Interaction of Cibacron Blue F_3GA with glutamine synthetase. Use of the dye as a conformational probe. 2. Studies with isolated dye fractions. Biochemistry 24: 661-666, 1985.

Stadtman, E. R. and Wittenberger, M. E.: Inactivation of Escherichia coli glutamine synthetase by xanthine oxidase, nicotinate hydroxylase, horseradish peroxidase or glucose oxidase: Effects of ferredoxin, putidaredoxin and menadione. Arch. Biochem. Biophys. 239: 379-387, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00212-14 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Ammonia-Assimilatory Enzymes in E. coli K12

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Mary Anne Berberich, Research Chemist, Laboratory of Biochemistry, NHLBI

Others: Sue Goo Rhee, Research Chemist, Laboratory of Biochemistry, NHLBI

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SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The term "nitrogen control" describes the phenomenon whereby a limitation of the ammonia supply during growth of bacteria results in an increase in the synthesis of ammonia-assimilatory enzymes, some amino acid binding proteins, and some amino acid catabolic enzymes. Genetic studies with enterobacteria reveal that regulation via nitrogen availability is under the control of three regulatory loci: glnF, glnG, and glnL. Both positive and negative controls operate at the transcriptional level where the products of glnF and glnL appear to mediate the interconversion of the glnG product, NR1, from repressor to positive activator in response to nutritional conditions. However, neither the biochemistry of NR1 activation nor the process by which the level of intracellular ammonia signals this interconversion is understood at present.

For this reason, a study of the physiological parameters of the nitrogen control response was made in E. coli K12 using the level of glutamine synthetase (GS) as a measure of regulation. It was determined that some D-amino acids are capable of imposing a step-down physiology upon cells growing in medium containing excess ammonia. Furthermore, these studies show that, added in combination at 10 mM each where growth rate is not appreciably affected, D-gln, D-thr, D-lys, and gly generate an increase in the level of synthesis of GS at a rate equivalent to that imposed by ammonia-limited growth.

The results of mutant sutdies indicate that adenyltransferase and pII play some role in the response to the D-amino acids whereas a positive response shows strict dependence upon the presence of the wild-type glnD allele. However, no correlation between a positive response and the state of adenylylation of GS can be made. Genetic experiments to assign map position to mutations affecting adenyltransferase and a unique constitutivity function have continued and the existence of genetic linkage between glnE and this gln "C" isolate has been excluded.

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Project Description:

Objectives:

1. To explore the hierarchy of nitrogen control in E. coli K12 and to study the interactions between the elements of the modification cascade which regulates GS activity and the genetic elements which govern GS synthesis using isogenic modification mutants and genetic regulatory mutants.

a) Determine physiological effectors of nitrogen control.

b) Determine response of regulatory mutants to physiological effectors of nitrogen control.

c) Identify other enzymatic components which may be involved in the positive activation process.

2. To conduct genetic experiments for the purpose of mapping mutations involved in nitrogen control in order to assist in clarifying their regulatory role.

a) Assign genetic location to the structural gene for adenyltransferase.

b) Locate the non-glnG,L, non-glnB constitutive mutation.

Methods Employed:

1. The Hierarchy of Nitrogen Control in E. coli K12 (N99).

a) Physiological effectors of nitrogen control. It has been determined that an increase in the level of GS can be provoked by the addition of either D-gln, D-lys, D-thr, or gly to cells growing in glycerol-mineral salts medium containing 100 mM ammonia nitrogen. No increase in specific activity was noted upon addition of any of the intermediates which participate in the modification cascade for GS activity regulation nor could an increase be observed when any of the allosteric inhibitors of GS activity were added to the culture. Identical results were observed whether or not cells had been permeabilized prior to addition of test compounds. The increase observed is independent of the function of GAT and GDH, and is eliminated when chloramphenicol is added along with the D-amino acids. When D-gln, D-lys, D-thr, and gly are added in combination, the rate of increase in GS level approximates that observed during step-down of a culture from ammonia excess to ammonia-limited growth conditions (Table 1). As observed upon exhaustion of the nitrogen in the growth medium, the increase in GS level provoked by addition of these D-amino acids occurs without a lag and is essentially complete within 60 min. Strain N99 does not utilize D-gln, D-lys, or D-thr as sole carbon or nitrogen source. Glycine can serve as sole nitrogen source but its utilization is markedly stimulated in the presence of L-glu.

Table 1. Survey of D-amino Acids and GS Level in Wild Type

Additions[a]	GS specific activity units/mg	Additions[a]	GS specific activity units/mg
None	0.29	D-serine	0.28
D-glutamate	0.46	D-threonine	0.42
D-valine	0.25	D-leucine	0.21
D-lysine	0.41	Glycine	0.43
D-alanine	0.28	D-cysteine	0.26
D-Øalanine	0.20	D-tyrosine	0.26
D-isoleucine	0.26	D-tryptophan	0.28
D-glu + D-lys + D-thr + gly[c]	0.81	D-glu + D-lys + D-thr + gly[b]	0.61
D-glu + D-lys + D-thr + gly[d]	0.88	Nitrogen limited growth[e]	0.99

[a]Individual amino acids were added at 10 mM. Additions were made to cells at mid-log growth in glycerol-mineral salts-ammonia (100 mM). Cells wer harvested after 60 min (Klett 100; 660 filter), washed with 0.85% NaCl and resuspended in imidazole (20 mM), $MgCl_2$ (10 mM), and KCl (100 mM) buffered at pH 7.3. Extracts were prepared by sonication and clarified by centrifugation for 15 min at 15,000 x g. Protein was determined by Lowry method.

[b]Total amino acids added = 10 mM; 2.5 mM each.

[c]Total amino acids added = 20 mM; 10 mM D-glu + 3.3 mM each D-lys, D-thr, gly.

[d]Total amino acids added = 40 mM; 10 mM each.

[e]Enzyme activity measured 60 min following step-down of culture from glucose (0.2%)-mineral salts-ammonium chloride (20 mM)-glutamine (3 mM) to glucose-mineral salts-glutamine (10 mM) growth medium.

b) Determination of the Response of Regulatory Mutants to Effectors of Nitrogen Control. Table 2 shows the response to D-amino acids of strains derived from E. coli K12 (N99) which are altered in various functions pertaining to nitrogen control. The constitutive glnG mutant produces an NR_1 which is apparently ineffective as a repressor and demonstrates the greatest amplitude of response to the addition of the D-amino acid mixture. The glnB (pII) and glnE (AT) mutants increase the level of GS in response to the mixture of D-amino acids but not to D-glu alone. The glnD (UT) mutant does not demonstrate an increase in GS in response to D-amino acids whether or not the cells are permeabilized prior to addition. This latter finding, along with the observation that glnD mutants are extremely sensitive to γ-glutamyl hydrazide, would suggest that the lack of response is not simply due to decreased transport. In addition, Sue Goo Rhee has assayed the purified UT/UR in the presence of D-amino acids and finds that none of these (D-gln, D-lys, D-thr, or gly), when added individually at 5 mM, affect either enzymic activity substantially. The effect of a combination of these has not yet been explored in this assay; however, another function for UT/UR in the process of regulation of synthesis cannot be ruled out.

c) Identification of Other Components Involved in Positive Activation.

i. Consistent with a physiologic role for the D-amino acids in nitrogen control is the finding that a specific glutamate racemase is elevated under nitrogen-limited growth (glutamine or glutamate as sole nitrogen source) as well as upon addition of D-glutamate to nitrogen sufficient cells. However, preliminary studies do not indicate differences in the level of this enzyme among the various regulatory strains following addition of D-glu. In addition, a glutamate dehydrogenase active with DCPIP at pH 6.5 was also elevated following addition of D-glu although regulatory mutants have not yet been compared with respect to their levels of this enzyme. No specific racemase or dehydrogenase could be demonstrated following addition of D-lys, D-thr, or gly, but some early studies indicate an elevation in glycine oxidase in these instances. It is possible that one or more of these catabolic processes is involved in the interconversion of NR_1 (repressor) to NR_1 (activator) at $glnAp_2$.

ii. That the level of GS in E. coli K12 (N99) is responsive to the carbon source as well as the nitrogen source supplied in the growth medium has been shown and the response of the various nitrogen regulatory mutants to growth on lactose-mineral salts-ammonia has been compared (1). Unlike the response to D-amino acid addition, growth on lactose C as compared to glycerol C leads to an increase in $\bar{n}$ as well as to an increase in GS level. The lowest incremental response was observed with the AT^- and gln "C" types where $\Delta\bar{n}$ is minimal. A glnAp mutation resulted in an increase in GS during growth on lactose while remaining unresponsive to both nitrogen limitation and D-amino acid addition. A bimodal form of regulation was suggested to explain the differences observed among the regulatory strains compared with respect to nitrogen or carbon response (1). Subsequent evidence from an analysis of transcripts produced under various nutritional conditions indicates the presence of two promoters for glnA; one responsive to cAMP and one

Table 2. Effect of D-amino Acids on Mutants Altered in GS Regulation

Strain	Relevant Genotype[a]	Level of Glutamine Synthetase[b] units/mg	$\bar{n}$	Increase GS Units after Additions D-glu[c]	Mix[d]
N99	relA$^+$	.33	3.5	.20	.59
N100	relA	.38	3.4	.17	.57
MB673	glnG	1.73	8.3	.24	1.56
MB1072	glnE	.32	1.0	.05	.36
MB1082	glnB	1.38	9.8	.03	.35
MB1	glnD[e]	.16	10.0	0	.04
MB1/ pLC38-39	glnD$^+$	.51	2.1	.19	N.D.[f]

[a]Function affected: relA, ppGpp regulation; glnG, regulation, nitrogen control; glnE, bifunctional adenylyltransferase-adenyl removing enzyme; glnB, P_{II} modifier protein of adenylyltransferase and substrate for UT/UR; glnD, bifunctional uridyltransferase-uridyl removing enzyme. 38-39, plc plasmid carrying glnD$^+$.

[b]Enzyme levels measured in cells harvested during log-phase growth in glycerol-mineral salts-ammonia (100 mM). Value for $\bar{n}$ included as part of strain description only; no change on D-amino acid addition.

[c]As [b], with addition of 10 mM D-glutamate 60' prior to harvest.

[d]As [b], with addition of 10 mM each, D-glutamate, D-lysine, D-threonine and glycine 60' prior to harvest.

[e]2 mM L-glutamine present as growth supplement. No effect on level of response to D-amino acid addition at this concentration of L-gln observed with wild type strain (data not shown).

[f]Not determined.

f5

responsive to nitrogen limitation (ref. 1). These results support the findings with the strain carrying the glnAp 141 mutation and suggest that the more precise designation is glnAp$_2$-141.

2. Genetic Mapping of Mutations Involved in Nitrogen Control.

a) Adenyltransferase. So far, no position for glnE can be assigned on the genetic map although > 50% of the chromosome has been examined by means of Hfr-mediated transformation selected to correct defects caused by tn 10 insertions into AT$^-$ mutant. Many anomalies continue to appear during evaluation of recombinants. A plasmid carrying AT, plc 18-28 (Carbon Collection), from which adenyltransferase was cloned, corrected a ptsI-cysK deletion at min 52. However, standard genetic methods do not indicate that glnE (AT) could be located between min 43-61. Also, a correction of the reduced level of GS measured in AT$^-$ strains is approximately 80% co-transducible with metE at min 86. However, no ability to increase $\bar{n}$ which would indicate the presence of adenyltransferase activity can be demonstrated in these recombinants. The current approach is described below.

b) Unique Constitutive Type Suppressor of glnD. Recently, the gln "C" mutation has been localized between min 56-61 by transformation. Determination of a more precise location by means of co-transduction frequencies with markers in this region is currently in progress.

Proposed Course:

1. Pursue the mechanism of the D-amino acid provocation of the positive nitrogen control response.

a) Will attempt chiral resolution of D and L gln, lys, thr, and gly for purposes of assessing their intracellular concentration before and after exhaustion of the ammonia supply in growth medium. Currently involved in preparing samples for analysis by gas chromatography performed in conjunction with Dr. P. E. Hare of the Carnegie Institute of Washington. Will continue to work on methods to minimize technical difficulties encountered in resolution from extraction mixtures.

b) Will explore the possiblity that a functional γ-glutamyl cycle generates the nitrogen regulatory signal for transcription at glnAp$_2$. It had earlier been shown in this work that addition of D-5-oxoglutamate (gift of A. Meister) but not D-L pyroglutamate (Sigma) provoked an increase in GS level. Pyroglutamate would be expected to be generated from glutathione (or glutamine) as a function of the γ-glutamyl cycle. Additional concentration studies with D-5-oxoglutamate and L-5-oxoglutamate recently purchased from Fluka Chemical can now be performed. It is known that the glutathione level in E. coli increases at maximum stationary phase (ref. 2). It will be of interest to determine whether it increases also upon nitrogen-limitation and whether D-glu, perhaps derived from re-entry of cell wall peptides (ref. 3) comprises a portion of the glu-cys-gly peptide. The ability of D-5-oxoglutamate to evoke a positive response from the glnD mutant is currently being tested.

2. Proceed with genetic analyses.

a) Adenyltransferase. In order to facilitate rapid screening for AT transformation, a pair of oppositely transferring Hfr's will be made polA in order to insure integration of transforming DNA into the chromosome by recombination. Cloned (AT) DNA containing an amp^r marker will then be used to transform these Hfr's. Subsequently, donation of the ATP gene to marked recipients will be monitored by means of the simultaneous transmission of ampicillin resistance according to standard mapping techniques (ref. 4). The polA Hfr's are currently being constructed.

b) gln "C" Mapping. Genetic markers in the 56-61 min interval will be used to generate a linkage map based on co-transduction frequencies.

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3. Goodell, E. W. and V. Schwarz. Release of cell wall peptides into culture medium by exponentially growing E. Coli. J. Bact.: 391-397, 1985.

4. Silver, P. and W. Wickner. Genetic Mapping of the E. coli leader peptidase gene: A new approach for determining the map position of a cloned gene. J. Bact. 154: 569-572, 1983.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00224-08 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Calcium-Calmodulin-Regulated Enzymes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others: Marina Lanciotti, Visiting Fellow, Laboratory of Biochemistry, NHLBI
(Appointment began November, 1984)
Aile Zhang, Visiting Fellow, Laboratory of Biochemistry, NHLBI
(Appointment began March, 1985)

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SECTION

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INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	2.3	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

(1) The mechanism of activation of calmodulin-dependent phosphoprotein phosphatase by a crucial divalent metal ion like Ni^{2+} or Mn^{2+} has been studied under diverse conditions. Activation of the phosphatase by Ni^{2+} is characterized by an initial lag period and by extremely tight binding. Different levels of Ni^{2+} give rise to the same final extent of activity, although the lag time decreases as Ni^{2+} concentration is raised. In the presence of calmodulin, only one Ni^{2+} ion is bound, the lag phase conforms with first-order kinetics, and the initial enzyme$\cdot Ni^{2+}$ complex is inactive. The initial dissociation constant for Ni^{2+} is 2.5 mM and the rate constant for the activation process (conformational rearrangement) is $0.083 s^{-1}$. In the absence of calmodulin, the lag phase no longer conforms with a first-order process. Both kinetic and binding experiments indicate the involvement of two Ni^{2+} ions. It appears that the additional Ni^{2+} binding site overlaps with the calmodulin binding domain on the phosphatase. Activation by Mn^{2+} in the presence of calmodulin is similar to that by Ni^{2+} except that the process is reversible. The initial enzyme$\cdot Mn^{2+}$ binding constant, 200 μM, is tighter, but the activation rate constant, $0.042 s^{-1}$, is somewhat slower compared with corresponding parameters for Ni^{2+}.

(2) The activation of calmodulin-regulated enzymes by Ca^{2+} was treated theoretically by assuming all the components involved were in rapid equilibria. But the extremely slow off-rate of calmodulin from the enzyme$\cdot$calmodulin complex seems to contradict the assumption. A mathematical derivation has been developed which demonstrates that the rapid-equilibrium assumption is valid as long as the binding of Ca^{2+} to various enzyme$\cdot$calmodulin complexes are rapid.

(3) The calmodulin-dependent phosphoprotein phosphatase was found to be phosphorylated by the Ca^{2+}-dependent C kinase. The phosphorylated form of calmodulin-dependent protein kinase is dephosphorylated by the calmodulin-dependent phosphatase.

Project Description:

Objectives:

(1) To gain knowledge of regulatory and catalytic mechanisms of enzymes mediated by Ca^{2+} and phosphorylation/dephosphorylation.

(2) To develop or improve methods and theories applicable to the study of enzymes.

Major Findings:

(1) Mechanism of activation of calmodulin-dependent phosphoprotein phosphatase by Ni^{2+} and Mn^{2+}. The calmodulin-dependent phosphoprotein phosphatase (CPP) requires a divalent metal ion such as Ni^{2+}, Mn^{2+}, Mg^{2+}, etc., for maintaining full activity and structural integrity. When Ca^{2+} is the only ion present, CPP is inactive in the absence of calmodulin (CaM); whereas in the presence of Ni^{2+} or Mn^{2+}, CPP is active without CaM although Ca^{2+}-CaM further stimulates CPP activity. The time course of activation is characterized by an initial lag time, signifying a slow conformational rearrangement. Key observations regarding the mechanism of activation are as follows:

(a) Previously, binding of Ni^{2+} to CPP was shown to be extremely tight by fluorescence studies in which the quenching observed upon addition of Ni^{2+} could not be reversed by metal chelators. The virtually irreversible binding of Ni^{2+} is further supported by the observation that different Ni^{2+} concentrations give rise to the same final extent of activation. The lag time, however, decreases as Ni^{2+} concentration is increased and is not affected by varying levels of the substrate, p-nitrophenylphosphate, used. Thus, the mechanism of Ni^{2+} activation of CPP in the presence of CaM can be described by a relatively simple scheme:

$$E \underset{}{\overset{K_N}{\rightleftharpoons}} EN \xrightarrow{k_1} E'N$$

where E denotes the CPP-CaM complex, N represents Ni^{2+}, K_N is the dissociation constant of Ni^{2+}, E'N is the activated EN form, and k_1 is the first-order rate constant for the activation process.

Integration of the above reaction scheme yields:

$$E'N = E_o\{1-\exp[-k_1Nt/(K_N+N)]\}$$

$$\text{and } EN = [N/(K_N+N)]E_o \cdot \exp[-k_1Nt/(K_N + N)]$$

Since, in the absence of the activating divalent metal ion, the CPP-CaM complex undergoes a time-dependent deactivation reaction, the E form is inactive (preincubated with Ca^{2+} and CaM) in our experiments, and the product formation curve has the expression:

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$$P = k_3E_ot+[k_3K_N+(k_3-k_2)N]E_o\{\exp[-k_1Nt/(K_N+N)]-1\}/k_1N$$

where k_2 and k_3 are the catalytic constants for the EN and E'N forms, respectively. At steady-state, we have:

$$P_{s.s.} = k_3E_ot-[k_3K_N+(k_3-k_2)N]E_o/k_1N$$

$$\text{and } \Delta P = P-P_{s.s.} = [k_3K_N+(k_3-k_2)N]E_o\cdot\exp[-k_1Nt/(K_N+N)]/k_1N$$

The first order activation process has been confirmed by ln ΔP vs. t plots which are linear up to three half-lives. The slopes of these plots, k_{obs}, are defined as:

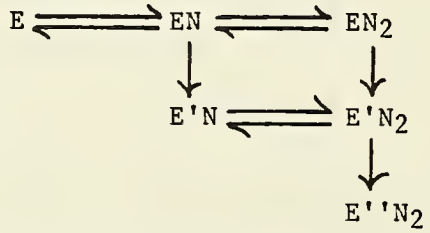
$$k_{obs} = k_1N/(K_N+N)$$

and then compared with the lag times, τ, determined from the intercept of the steady-state product formation line on the time axis:

$$\tau = (K_N+N)/k_1N-(k_2/k_1k_3) = (1/k_{obs})-(k_2/k_1k_3)$$

Comparison of τ and $1/k_{obs}$ obtained from the same experiments at different levels of Ni^{2+} reveals that they usually agree quite well, indicating that $k_2 \to 0$, i.e., the EN form is inactive. If the EN form is catalytically active, the values of τ should be consistently smaller than $1/k_{obs}$. When τ is plotted against 1/N, a linear line is obtained which is in agreement with the proposed mechanism. From the plot, K_N is estimated to be 2.5 mM and k_1 is $0.063s^{-1}$. It is clear that one Ni^{2+} ion initially combines with the CPP-CaM complex rather weakly and the Ni^{2+}-CPP-CaM complex then slowly converts to an activated form.

(b) In the absence of CaM, Ni^{2+} also activates CPP in an irreversible manner. Kinetic analysis, however, shows that the lag phase does not conform with a first-order process. The τ vs. 1/N plot yields a nonlinear line that is concave downward. Binding experiments using $^{63}Ni^{2+}$ and Sephadex G-50 column indicate that 1.7 moles of Ni^{2+} are bound per mole of CPP. Other laboratories have reported a similar number of bound Ni^{2+}. Therefore, existing evidence supports that at least two Ni^{2+} ions are bound and more than one conformational step is involved. The simplest mechanism that can describe the observed kinetic process is:



It seems that the different activation patterns observed in the presence and absence of CaM are due either to CaM hindering the binding of the

90

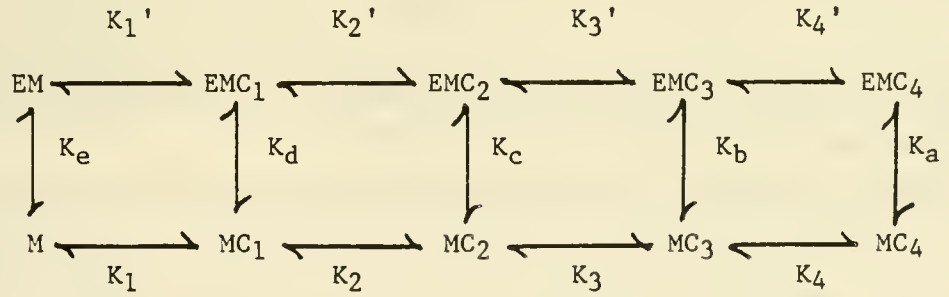
second Ni^{2+}, or to overlapping of CaM binding domain with the second Ni^{2+} binding site.

(c) The activation of CPP by Mn^{2+} is less dramatic, the resultant stimulation being ~1/20 of that caused by Ni^{2+}. Basically, in the presence of CaM the mechanism of activation by Mn^{2+} is similar to the Ni^{2+} case except that the process is not irreversible (M = Mn^{2+}):

$$E \underset{}{\overset{K_o}{\rightleftharpoons}} EM \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E'M$$

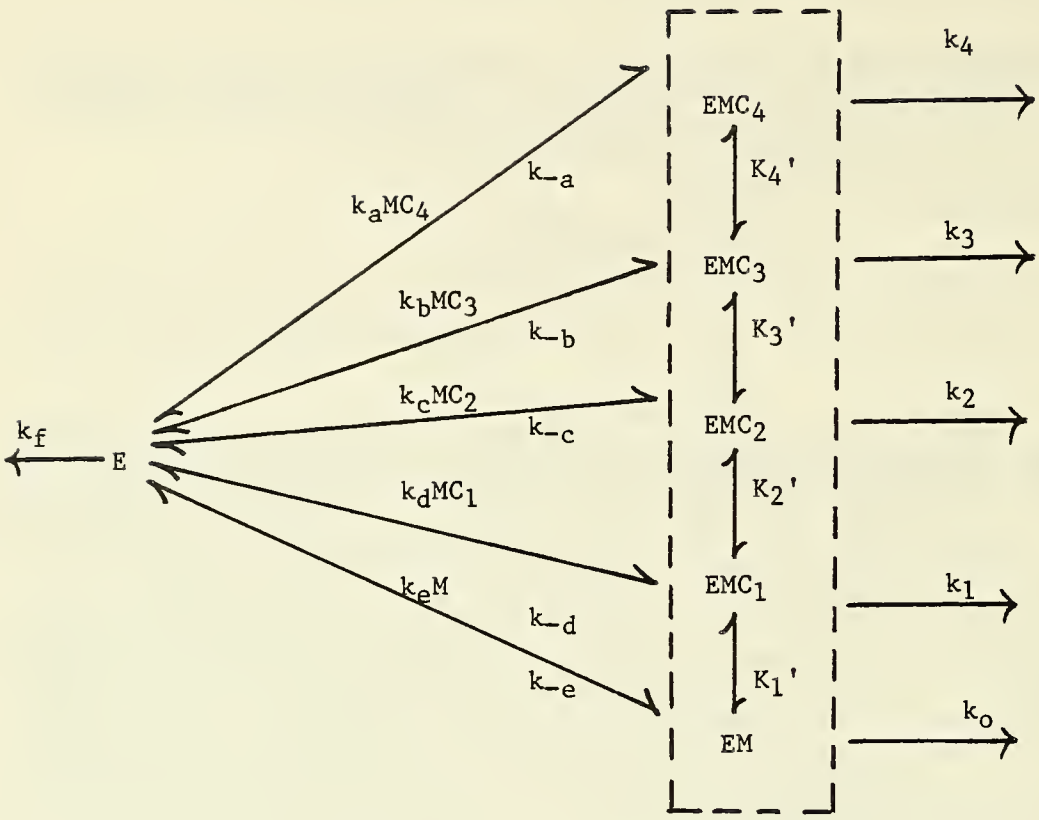
K_o and k_1 obtained from τ vs. 1/M plots are 200 μM and $0.042s^{-1}$, respectively.

(2) Validity of rapid equilibrium treatment for the Ca^{2+} activation of CaM-regulated enzymes. The activation of cyclic nucleotide phosphodiesterase (PDE) by Ca^{2+} and CaM has been treated theoretically as a system in which the components involved--PDE, CaM, and Ca^{++}--are in rapid equilibration (Scheme 1). We have shown in a previous report that, while the on-rate constant for the combination of PDE and CaM·Ca_4^{2+}, ~4 X $10^6 M^{-1}s^{-1}$, is reasonably fast, the off-rate constant, 3.2 X $10^{-3}s^{-1}$, is extremely slow. Thus, it appears that rapid equilibrium treatment may not apply to the PDE or similar CaM-regulated enzyme systems. It can be shown, however, that as long as the on-off rates of Ca^{2+} are fast (that is, the binding of Ca^{2+} to CaM and to various E·CaM complexes can be treated as in rapid equilibria), a mathematical expression identical to that derived from rapid-equilibrium treatment can be obtained by applying the more stringent steady-state assumption to the binding of E and various CaM-Ca^{2+} complexes (Scheme 2).



Scheme 1

M = CaM; C = Ca^{2+}, the subscripts indicate the number of Ca^{2+} ions bound; the K's are dissociation constants.



Scheme 2

k_a, k_{-a}... etc. are the on- and off-rate constants for various E·CaM complexes; k_o, k_1, k_2, k_3, k_4, k_f are catalytic constants. Area enclosed by dotted lines denotes the rapid-equilibrium segment.

Combined steady-state and equilibrium treatment according to Scheme 2 yields:

$$\frac{\Delta v}{E_t} = \frac{v-v_f}{E_t} = \frac{(k_o f_o+k_1 f_1+k_2 f_2+k_3 f_3+k_4 f_4-k_f)(k_e M+k_d MC+k_c MC_2+k_b MC_3+k_a MC_4)}{(k_{-e} f_o+k_{-d} f_1+k_{-c} f_2+k_{-b} f_3+k_{-a} f_4)+(k_e M+k_d MC+k_c MC_2+k_b MC_3+k_a MC_4)}$$

where f_o, f_1, f_2, f_3, f_4 are the fractions of EM, EMC, EMC_2, EMC_3, EMC_4 in the equilibrium segment, e.g., $f_4 = C^4/K_1'K_2'K_3'K_4'\phi_2$ ($\phi_2 = f_o+f_1+f_2+f_3+f_4$). Since the E·CaM·$Ca_4^{2+}$ complex formation is diffusion-controlled, conceivably all the on-rate constants for the E·CaM complexes are the same in view of the fact that the sizes of the CaM·Ca^{2+} complexes are, in essence, unchanged, i.e., $k_a = k_b = k_c = k_d = k_e$. It follows that

$$\frac{\Delta v}{E_t} = \frac{(\Delta k_o+\Delta k_1 C/K_1'+\Delta k_2 C^2/K_1'K_2'+\Delta k_3 C^3/K_1'K_2'K_3'+\Delta k_4 C^4/K_1'K_2'K_3'K_4')M_t}{[(K_e f_o+K_d f_1+K_c f_2+K_b f_3+K_a f_4)+M_t]\phi_2}$$

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Substitution of the relationships defined in Scheme 1:

$$\frac{K_a}{K_1'K_2'K_3'K_4'} = \frac{K_e}{K_1K_2K_3K_4}, \quad \frac{K_b}{K_1'K_2'K_3'} = \frac{K_e}{K_1K_2K_3}, \text{ etc.,}$$

into the above equation converts $(K_ef_o+K_df_1+K_cf_2+K_bf_3+K_af_4)$ into $K_e\phi_1/\phi_2$ $(\phi_1=1+C/K_1+C^2/K_1K_2+C^3/K_1K_2K_3+C^4/K_1K_2K_3K_4)$, and reduces the equation to:

$$\frac{\Delta v}{E_t} = \frac{M_t\left[\Delta k_o+\Delta k_1\frac{C}{K_1'}+\Delta k_2\frac{C^2}{K_1'K_2'}+\Delta k_3\frac{C^3}{K_1'K_2'K_3'}+\Delta k_4\frac{C^4}{K_1'K_2'K_3'K_4'}\right]}{K_e\left(1+\frac{C}{K_1}+\frac{C^2}{K_1K_2}+\frac{C^3}{K_1K_2K_3}+\frac{C^4}{K_1K_2K_3K_4}\right)+M_t\left(1+\frac{C}{K_1'}+\frac{C^2}{K_1'K_2'}+\frac{C^3}{K_1'K_2'K_3'}+\frac{C^4}{K_1'K_2'K_3'K_4'}\right)}$$

where $\Delta k_4 = k_4-k_f$, $\Delta k_3 = k_3-k_f$, etc. This expression is identical with the rate equation derived from rapid-equilibrium assumption, demonstrating the validity of previous kinetic treatment.

(3) In collaboration with Dr. Patel of NIMH, the structure and function of CPP in relation to phosphorylation/dephosphorylation have been investigated. Preliminary findings are:

(a) CPP was found to be phosphorylated by this laboratory. Although the cyclic AMP-dependent protein kinase can phosphorylate CPP, the reaction was slow. The Ca^{2+}-dependent C kinase, however, was found to be capable of phosphorylating CPP rapidly. The phosphorylation is dependent on the presence of Ca^{2+}, phospholipid, and is stimulated by phorbol esters. The incorporation of phosphate was approximately one per mole of CPP.

(b) The calmodulin-dependent protein kinase was found to be a good substrate for CPP.

The above findings further point to the intriguing interplay involving Ca^{2+} regulation and phosphorylation/dephosphorylation.

Proposed Course:

(1) Mechanism of activation of CPP by Ca^{2+}; effect of phosphorylation of CPP on its function.

(2) Isolation and characterization of the Ca^{2+}-sensitive phosphatase.

(3) Interactions among CPP, C kinase, and calmodulin-dependent protein kinase.

Publications:

Huang, C.Y. and King, M.M.: Mode of Ca^{2+} activation of calmodulin-regulated enzymes. Curr. Top. Cell. Regul., in press, 1985.

King, M.M., Lynn, K.K., and Huang, C.Y.: Activation of the calmodulin-dependent phosphoprotein phosphatase by nickel ion. In Brown, S.S. and Sunderman, F.W., Jr. (eds.): Progress in Nickel Toxicology. Oxford, England, Blackwell, in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00225-08 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mixed-Function Oxidation of Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Rodney L. Levine, Senior Investigator, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

Department of Microbiology and Molecular Genetics
Harvard Medical School
Boston, Massachusetts

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues X☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Many enzymes are now known to be subject to a covalent modification mediated by mixed-function oxidation. This modification may have diverse physiologic and pathologic significance. This includes the regulation of protein turnover, accumulation of modified proteins during aging, killing of pathogens by host defense mechanisms, limitation of autolysis, and oxygen toxicity.

The role of oxidative modification in marking proteins for turnover is supported by the purification of a protease from E. coli which specifically degrades oxidatively modified glutamine synthetase. The unmodified protein is not a substrate.

Multiple oxidative modifications may be introduced into a protein. Glutamine synthetase was subjected to varying times of exposure to mixed-function oxidation to provide samples of graded oxidation. The enzyme lost catalytic activity due to oxidation of a specific histidine residue. However, susceptibility to proteolysis did not correlate with this oxidation. Following a lag period, a second histidine residue was oxidized. The time course of this modification did match that of proteolytic susceptibility. The enzyme underwent other changes during modification, including exposure of sulfhydryl residues, weakening of subunit interaction, formation of aggregates, and decreased thermal stability.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives: Previous years' reports described a covalent modification of glutamine synthetase which is mediated by various mixed-function oxidation systems. Many enzymes and structural proteins now appear susceptible to such oxidations. The physiologic functions remain to be determined, but the modification does appear to "mark" the protein for subsequent proteolytic degradation. The objectives of this project are determination of the chemical nature of the modification; purification and characterization of the enzymes which catalyze the modification and subsequent proteolysis; and assessment of the physiologic controls which regulate the modification and proteolysis of specific proteins.

Major Findings:

1. Purification of an E. coli protease which selectively degrades oxidized glutamine synthetase. Earlier studies suggested that intracellular turnover of glutamine synthetase is a two-step process. In the first step, the enzyme is oxidatively modified. In the second step, the modified protein is degraded by a protease. This scheme requires protease(s) which selectively degrade the oxidatively modified protein, while sparing the native enzyme. Such a protease has been purified to homogeneity from extracts of E. coli. It is a monomeric protease (MW = 75,000) which readily degrades oxidatively modified glutamine synthetase; native glutamine synthetase is not a substrate. The proteolytic cleavage occurs initially near the carboxy terminus of the protein with release of 46,000 and 4,000 MW products. The insulin B chain and casein are also substrates, but hemoglobin is not.

2. Multiple oxidative modifications may be introduced into a protein. Exposure of many enzymes to mixed-function oxidation causes loss of catalytic activity. In the case of glutamine synthetase, this inactivation is due to oxidation of a specific histidine residue (His*):

-Met-His*-Cys-His-Met.

This peptide probably forms the metal-nucleotide binding pocket at the catalytic site. When exposed just long enough to inactivate the enzyme, the modified glutamine synthetase has increased susceptibility to proteolytic digestion. However, additional exposure to the mixed-function oxidation greatly enhanced the susceptibility to proteolysis. This result implied that additional changes occurred on longer oxidation.

Study of the time course of oxidation revealed multiple changes which followed different time courses. All required continued exposure to the oxidizing systems; they did not result from secondary changes which could follow the initial oxidation.

As noted, inactivation and loss of a histidine residue occurred with initial exposure. Other characteristics were changed more slowly and were approximately proportional to the time of oxidation: exposure of sulfhydryl groups; weakening of subunit interactions; conversion to the "relaxed" confor-

mation; increased aggregation; and decreased thermal stability. As noted in earlier reports, carbonyl groups are also introduced into the protein. This reaction approaches a plateau with one carbonyl group per subunit.

None of these changes matched the pattern of susceptibility to proteolysis. This pattern shows a lag phase, followed by rapid increase in susceptibility, and then a plateau. Amino acid analyses detected only two changes, the loss of the first histidine residue followed by the loss of a second histidine. The time course of oxidation of the second histidine matches that for proteolysis. Thus, "marking" of glutamine synthetase for proteolytic digestion requires the mixed-function oxidation of two histidine residues.

3. Glycerol dehydrogenase undergoes oxidative modification in vivo. In Klebsiella, glycerol may be metabolized through an anaerobic pathway via glycerol dehydrogenase, or through an aerobic pathway via glycerol kinase. Exposure to oxygen causes rapid inactivation of the glycerol dehydrogenase as well as other dehydrogenases involved only in anaerobic metabolism. The characteristics of these inactivations suggested that they were mediated by mixed-function oxidation. We have prepared "inactivated" glycerol dehydrogenase in vitro by exposing the enzyme to mixed-function oxidation by an ascorbate/oxygen system. The properties of this modified enzyme were compared to those of the enzyme isolated from cells exposed to oxygen. The in vitro and in vivo inactivated enzymes appear identical. In particular, both demonstrate increased K_m for NAD; an increase in NAD concentration from 1 to 10 mM restored activity of both in vitro and in vivo oxidized enzymes.

It is notable that the oxidatively modified dehydrogenase is apparently degraded very rapidly in vivo. Thus, oxidative modification of glycerol dehydrogenase also marks the protein for degradation.

4. Additional findings. The introduction of carbonyl groups provides the basis for a presumptive assay of oxidative modification. Reduction of the carbonyl by tritiated borohydride labels the protein with tritium. Modifications in this assay now permit the quantitation of carbonyl content using as little as 10 micrograms of protein.

Oxidatively modified bovine and yeast superoxide dismutases and bovine albumin were provided by other laboratories. The superoxide dismutases were not susceptible to proteolysis by either the E. coli or liver proteases. However, modified albumin was preferentially degraded by both proteases.

Significance to Biomedical Research and the Program of the Institute:

Studies in several laboratories provide a growing list of proteins which are susceptible to oxidative modification. The isolation of mammalian and bacterial proteases which are specific for the modified proteins supports our earlier suggestion that the modification serves as a marker for intracellular protein turnover. Regulation of the oxidative modification provides control of the turnover of specific proteins. In addition, the modification may be important in switching from anaerobic to aerobic metabolism, in host defense mechanism, in limitation of the inflammatory response, in the accumulation

of altered proteins in aging, and in oxygen toxicity (including bronchopulmonary dysplasia, adult respiratory distress syndrome, retrolental fibroplasia, and in reperfusion-mediated ischemic damage).

Proposed Course:

Work will continue on glutamine synthetases with varying degrees of oxidative modification. These studies permit definition of the chemical nature of the modifications and of the effect of the modifications, especially on susceptibility to proteolysis. The potential functions of oxidative modification will be explored in several systems: The protease(s) from E. coli will be studied in more detail, with attention to defining natural and artificial substrates. The rapid inactivation and degradation of glycerol dehydrogenase of K. aerogenes provides a system to study both proteolysis and the role of oxidative modification in coordinating the switch from anaerobic to aerobic metabolism. Study of in vitro basement membrane proteins as well as animal models of respiratory distress may implicate oxidative modification in pulmonary oxygen toxicity.

Publications:

Johnson, E.A., Levine, R.L., and Lin, E.C.C.: Inactivation of glycerol dehydrogenase of Klebsiella pneumoniae and the role of divalent cations. Journal of Bacteriology, in press, 1985.

Levine, R.L.: Mixed function oxidation of proteins. Current Topics in Cellular Regulation 27: in press, 1985.

Oliver, C.N., Ahn, B., Wittenberger, M.E., Levine, R.L. and Stadtman, E.R.: Age-related alterations of enzymes may involve mixed-function oxidation reactions. In Adelman, R.C. and Rothstein, M., eds., Review of Biological Research on Aging, Alan R. Liss, New York, in press, 1985.

Moss, J., West, R.E., Jr., Osborne, J.C., Jr. and Levine, R.L.: Characterization of NAD: Arginine Mono-ADP-ribosyltransferases in turkey erythrocytes: Determinants of substrate specificity. In Seventh International Symposium on ADP-Ribosylation Reactions, Springer-Verlag, Berlin, in press, 1985.

Sliwkowski, M.X. and Levine, R.L.: Labelling of cysteine-containing peptides with 2-nitro-5-thiobenzoic acid. Analytical Biochemistry 147: 369-373, 1985.

Rivett, A.J., Roseman, J.E., Oliver, C.N., Levine, R.L. and Stadtman, E.R.: Covalent modification of proteins by mixed-function oxidation: Recognition by intracellular proteases. Progress in Clinical and Biological Research 180: 317-328, 1985.

Oliver, C.N., Fulks, R., Levine, R.L, Fucci, L., Rivett, A.J., Roseman, J.E., and Stadtman, E.R.: Oxidative inactivation of key metabolic enzymes during aging. In Roy, A.K. and Chatterjee, B., eds., Molecular Basis of Aging, Academic Press, New York: 235-260, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00237-06 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Toxicity and Transport of Bilirubin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Rodney L. Levine, Senior Investigator, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

Laboratory of Neurosciences
National Institute on Aging

Small Animal Section
Veterinary Resources Branch

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hyperbilirubinemia is probably the most frequently diagnosed and treated condition in the human newborn. Treatment is aimed at preventing the entry of bilirubin into the brain because of the risk of permanent neurologic damage. The mode by which bilirubin enters the brain, its metabolic fate after entry, and the sites of toxic action are unknown. Using osmotic stress, the blood-brain barrier was opened in rats, permitting entry of bilirubin. However, in this acute model, the bilirubin was rapidly cleared, with a half-time of 1.6 hours. To provide chronic exposure, especially in newborn animals, we are now studying two inbred strains of rats which lack glucuronyl transferase.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives: Bilirubin is an end-product of heme metabolism in humans. In vitro, bilirubin is toxic to cells and to isolated mitochondria. The biochemical basis of the toxicity is unknown. In vivo, bilirubin is remarkably benign, with the notable exception of newborns. While most infants suffer no lasting sequelae, a few develop bilirubin encephalopathy, or kernicterus. The mode of entry of bilirubin into the brain and the site of toxic action are unknown. The objective of this study is to understand these mechanisms of transport and the biochemical basis of its toxicity.

Major Findings:

Previous annual reports described attempts to develop a model of kernicterus utilizing osmotic opening of the blood-brain barrier. After opening, bilirubin entered the rat brain, and its distribution was similar to that seen in human kernicterus. However, pharmacokinetic studies demonstrated that this bilirubin was rapidly cleared from the brain, with apparent first-order kinetics and a half-time of 1.6 hours. Such clearance makes it difficult to study the biochemical effects of bilirubin on cerebral metabolism.

In attempting to develop other models, we explored use of the congenitally jaundiced rat (Gunn strain). The Gunn strain lacks glucuronyl transferase and develops unconjugated hyperbilirubinemia in the neonatal period. It provides a model for human neonatal jaundice. Dr. Carl Hansen has bred the Gunn strain into two defined, congenic strains: RHA and ACI. Surprisingly, only one of the two strains shows neurologic symptoms and death in the homozygous offspring. The other survives, apparently intact. While there have not yet been any measurements of the serum bilirubin levels, both strains appear quite jaundiced. Thus, absence of glucuronyl transferase causes severe neonatal jaundice in both strains, but only one is affected by the toxicity of bilirubin.

Significance to Biomedical Research and the Program of the Institute:

Current treatment of neonatal hyperbilirubinemia is generally based on the assumption that bilirubin is toxic per se. The two inbred rat strains provide animal models for examining the toxicity of bilirubin. The very different outcomes of the two strains demonstrate that there must be another factor which modulates the neurotoxicity of bilirubin.

Proposed Course:

The developmental pattern of the two strains will be defined, including pre- and post-natal concentrations of bilirubin, albumin, and unbound bilirubin as well as liver glucuronyl transferase activity. In collaboration with the Armed Forces Institute of Pathology, we will study the neuropathology of the two strains.

Publication:

Levine, R.L., Fredericks, W.R., Rapoport, S.I.: Clearance of bilirubin from rat brain after reversible osmotic opening of the blood-brain barrier. Pediatric Research 19: in press, October, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00239-06 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Glutamine Synthetase in E. coli and Yeast

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Sue Goo Rhee, Research Chemist, Laboratory of Biochemistry, NHLBI

Others: Won Gi Bang, Visiting Fellow, LB, NHLBI, 10/01/84 - 07/30/85
Kanghwa Kim, Visiting Fellow, LB, NHLBI, 10/01/84 - 09/30/85
Ja Hyun Koo, Guest Worker, LB, NHLBI, 10/01/84 - 02/28/85
P. Boon Chock, Chief, Section on Metabolic Regulation, LB, NHLBI
E. R. Stadtman, Chief, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.6	3.3	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Simple procedures involving an affinity chromatography step were devised to purify adenylyltransferase and uridylyltransferase from crude extracts of E. coli strain which had been engineered to overproduce them.

2. The mechanisms by which glutamine, α-ketoglutarate, and nucleotide monophosphate affect the uridylylation-deuridylylation reactions were studied in detail. In addition, kinetic parameters for these two opposing reactions were determined.

3. The variation in the concentration of uridylyltransferase and adenylyltransferase influences the rate of synthesis of GS in vivo. Studies with strains harboring a multicopy plasmid indicate that the elevated synthesis of the converter enzymes causes the enhanced GS synthesis in E. coli when grown on a nitrogen excess medium. The parallel relationship between the converter enzymes and GS seems to be due to the fact that the concentration of unbound P_{II}, which is required to repress the synthesis of GS, is dependent on the concentration of converter enzymes.

4. Many proteins, represented by yeast glutamine synthetase, E. coli adenylyltransferase, rabbit muscle pyruvate kinase, and E. coli glutamine synthetase are degraded in the presence of DTT and catalytic amounts of iron salts. This process requires oxygen, and the role of DTT and iron can be replaced by ascorbate and copper, respectively. Experimental data suggest that reactive oxygen species, likely hydroxyl radicals, generated locally around iron bound at the specific sites of enzymes are responsible for the degradation. In view of this finding, caution must be taken to prevent this type of oxidative modification when an enzyme is exposed to DTT because many biochemicals are contaminated with metal salts in sufficient quantities for catalyzing the formation of hydroxyl radicals.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives:

1. To study the role of two bifunctional enzymes, adenylyltransferase and uridylyltransferase in the regulation of glutamine synthetase in E. coli.

2. To understand the molecular mechanism of posttranslational regulation of glutamine synthetase in S. cerevisiae.

3. To study the chemical mechanism of protein cleavage in the presence of DTT and iron.

Major Findings:

1. The converter enzymes, adenylyltransferase and uridylyltransferase, catalyze the interconversion between unmodified and modified forms of glutamine synthetase and P_{II} protein, respectively. In an effort to obtain these two converter enzymes in large quantities, the glnE gene (the structural gene of adenylyltransferase) and glnD gene (the structural gene of uridylyltransferase) were cloned into a plasmid vector carrying the strong, regulatable λ phage promoter such that their synthesis was enhanced by several hundred fold. Homogeneous preparations of these two enzymes were obtained by simplified purification procedures involving two column chromatography steps; DEAE column chromatography followed by a Matrex gel (Amicon) dye affinity chromatography. Chromatography on the red gel for ATase and the green gel for UTase was the key procedure.

Purified UTase, albeit a single band when analyzed on SDS-polyacrylamide gel, gives two peaks, one for the monomeric form and the other for the oligomeric form having molecular weight larger than hexamer. The two forms show similar kinetic properties for the uridylylation reaction, but different properties for the deuridylylation reaction. The uridylylation reaction requires two substrates, P_{II} and UTP, and is stimulated by ATP and α-Kg, and is inhibited by glutamine. For the deuridylylation reaction, $P_{II}(UMP)_4$ is the only substrate, glutamine is an activator, and nucleotide monophosphates are potent inhibitors. The mechanism by which the effectors exert their effect on the uridylylation-deuridylylation reactions were studied in detail. The results are summarized in Tables 1 and 2.

In the uridylylation reaction, the binding of UTP is prerequisite for the binding of P_{II}. ATP is an essential activator (i.e., in the absence of ATP, no uridylylation reaction takes place) while the α-Kg activates the reaction by increasing both the $\overline{V}_{max}$ (21-fold) and the affinity of ATP (9-fold). Glutamine is a noncompetitive inhibitor with respect to P_{II}. The requirement of ATP as an activator is very strict; other analogs such as GTP, CTP, ADP, N_3-ATP, Aza-ε-ATP, and AMPPNP failed to activate.

Table 1. Kinetic properties of the uridylylation reaction at 28°, pH 7.0.

$\overline{V}_{max}$	+ α-Kg	0.29 μmole/min/mg
	- α-Kg	0.014 μmole/min/mg
$K_m(P_{II})$		17 μM
$K_m(UTP)$		11 μM
$K_a(ATP)$	+ α-Kg	8 μM
	- α-Kg	71 μM
$K_a(\alpha\text{-Kg})$	+ ATP	6 μM
	- ATP	40 μM
$K_i(gln)$		70 μM

In the deuridylylation reaction (Table 2), glutamine increases $\overline{V}_{max}$ and the affinity of $P_{II}(UMP)_4$ by 7- and 4-fold, respectively; while α-Kg has no effect. CMP inhibits by competing with $P_{II}(UMP)_4$.

Table 2. Kinetic properties of monomeric and oligomeric forms of UT-UR in the deuridylylation reaction at 28°, pH 7.0.

		$\overline{V}_{max}$ μmole/min/mg	$K_m(P_{IID})$ μM	$K_a(Gln)$, mM when $[P_{IID}]$=5.4 μM	$K_i(CMP)$, mM
monomer	+ Gln	1.15	0.6	1.3	0.2
	- Gln	0.16	2.2		
oligomer	+ Gln	0.88	1.1	1.2	2.2
	- Gln	0.41	2.3		

2. The posttranslational regulation of GS in E. coli involves three other proteins: regulatory protein P_{II}, ATase, and UTase. Although GS can be measured accurately, quantitative estimation of P_{II}, ATase, and UTase in crude

extracts are difficult. This is because these proteins exist in very low concentrations and because many metabolites in crude extracts affect the assays. In addition, P_{II} assumes two forms, unmodified and modified. To circumvent this problem, sheep antibodies were prepared against all four antigens and E. coli K-12 was grown on a nitrogen-limiting medium containing ^{35}S-methionine. Each component of the GS regulation cascade was precipitated with antibodies from the crude extracts and separated from impurities on the SDS-polyacrylamide gel. Proper protein bands were sliced, dissolved, and counted for their ^{35}S-radioactivity. Knowing the number of methionine in each protein, the molar ratio of UTase to ATase to P_{II} (tetramer) to GS (dodecamer) was calculated to be 1:2.6:11:34.

3. An elevated level of either of two converter enzymes due to the presence of multicopy plasmid has effect on the synthesis of GS in vivo. As shown in Table 3, the GS level in the strain harboring glnD or glnE is higher than that of wild type strain by 4- and 3-fold, respectively, when cells were grown on a nitrogen excess minimal medium (Table 3). Previously, it was suggested that the repression of GS synthesis in E. coli requires the presence of unmodified P_{II} protein. The concentration of unbound P_{II} is probably dependent on the amounts of two converter enzymes, uridylyltransferase and adenylyltransferase, for which P_{II} is a substrate and a modulator, respectively. In this connection we reasoned that the variation in the concentration of two converter enzymes changes the free P_{II} concentration and accordingly affects the GS synthesis. This hypothesis is consistent with the observation that the absence of P_{II} protein (due to mutation in the structural gene glnB) completely disengages control of the GS synthesis from the effects of uridylyltransferase and adenylyltransferase (Table 3).

4. During the storage in a buffer containing DTT, yeast GS degraded gradually. For example, upon incubation at 30° with a buffer containing 50 mM imidazole, pH 7.0, and 10 mM DTT, the yeast GS loses > 50% activity after 2 hr. Analysis of the 2 hr incubation sample on an SDS-polyacrylamide gel indicates that significant amounts of the 45K subunit peptide is cleaved into several peptides with MWs of 37K, 31K, and < 9K. Neither the addition of various protease inhibitors nor further purification on several HPLC columns had any effect on the rate of degradation. It seemed, therefore, unlikely that contaminating proteases are responsible. This observation led us to look for possible chemical cleavage of GS and other proteins. The damage reaction required oxygen and a reducing equivalent such as DTT or ascorbate. But the addition of EDTA or the Chelex treatment of all chemical reagents used prevented the damage reaction. In agreement with this observation, the addition of iron or copper salts increased the rate. Experimental data suggest that reactive oxygen species generated locally around metal ions bound at the specific site of GS are responsible for the degradation. Several other proteins were also subjected to the oxidative degradation. In the presence of 20 μM Fe^{+3} and 10 mM DTT, E. coli adenylyltransferase, E. coli GS, and rabbit muscle pyruvate kinase are easily cleaved. However, the cleavage of bovine serum albumin, ovalbumin, and A. niger glucose oxidase took place very slowly even in the presence of 100 μM Fe^{3+} and 10 mM DTT, and rabbit muscle aldolase was completely resistant to the cleavage reaction.

Table 3. Glutamine Synthetase Activities of Strains Harboring Multicopy Plasmids pglnD or pglnE[a]

gln genotype[b]/plasmid	Growth conditions	
	Nitrogen limiting[c]	Nitrogen excess[d]
wild type	0.84	0.09
wild type/pglnD7	0.89	0.36
wild type/pglnE10	0.85	0.29
glnB	0.73	0.46
glnB/pglnD7	0.88	0.46
glnB/pglnE10	0.73	0.46

[a]Activity of both adenylylated and unadenylylated subunits was measured by the γ-glutamyltransferase assay.

[b]Wild type strain was GP8551 and the strain lacking P_{II} protein was derived from GP8551 by inserting Mud1 into the glnB gene. Both strains were generously provided by Dr. Magasanik.

[c]Minimal medium with 4 mM glutamine and 0.5% glucose.

[d]Minimal medium with 4 mM glutamine, 20 mM ammonium chloride, and 0.5% glucose.

Proposed Course:

1. The bicyclic glutamine synthetase cascade system will be reconstituted in vitro by mixing purified components--UTase, ATase, P_{II}, and GS--in concentration identical to that of in vivo, and the property of this unique system will be studied.

2. The sequence of the glnB gene (structural gene of P_{II}) will be established.

3. The mechanism to inactivate yeast GS in response to glutamine shock will be studied. The amino acid composition and peptide mapping of inactive and active GS will be compared.

Publications:

Rhee, S.G., Chock, P.B., and Stadtman, E.R.: Nucleotidylation involved in the regulation of glutamine synthetase in E. coli. In Freedman, R. (Ed.): Enzymology of Posttranslational Modification of Proteins. New York, Academic Press, Vol. II, 1984, pp. 273-297.

Chung, H.K. and Rhee, S.G.: Separation of glutamine synthetase species with different states of adenylylation by chromatography on monoclonal anti-AMP antibody affinity columns. Proc. Natl. Acad. Sci. U.S.A. 81, 4677-4681, 1984.

Chung, H.K., Park, S.C., and Rhee, S.G.: Conformation-specific monoclonal antibodies to E. coli glutamine synthetase. J. Biol. Chem. 259, 11756-11762, 1984.

Rhee, S.G., Park, S.C., and Koo, J.H.: The role of adenylyltransferase and uridylyltransferase in the regulation of glutamine synthetase in E. coli. Curr. Topics Cell. Regul., in press, 1985.

Chock, P.B., Shacter, S., Jurgensen, S.R., and Rhee, S.G.: Cyclic cascade systems in metabolic regulation. Curr. Topics Cell. Regul., in press, 1985.

Rhee, S.G., Chock, P.B., and Stadtman, E.R.: E. coli glutamine synthetase. Meth. Enzymo. 103: 213-241, 1985.

Shacter, E., Chock, P.B., Rhee, S.G., and Stadtman, E.R.: Cyclic cascades and metabolic regulation. In Krebs, E. and Boyer, P. (eds.): The Enzymes. Vol. 18, in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00241-06 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Detection and Regulation of Phosphotyrosine Modification in Cellular Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Todd M. Martensen, Guest Worker, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

Laboratory of Vision Research, NEI
Laboratory of Cellular Metabolism, NHLBI
Diabetes Branch, NIADDK

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Tyrosine phosphate (Tyr-P) covalently attached to albumin potently induced the formation of sheep antibodies (Ab) which bound [14C]Tyr-P. Affinity purified Tyr-P Ab binding to [14C]Tyr-P was not inhibited by tyrosine sulfate, Ser-P, and Thr-P AT 50-100 fold the concentration of Tyr-P required for ~50% occupancy. The specificity of anti•Tyr-P Ab for Tyr-P residues in proteins was demonstrated in several ways. (1) Anti•Tyr-P Ab protected Tyr-P residues in proteins from dephosphorylation by phosphatases. (2) Anti•Tyr-P Ab quantitatively precipitated phosphotyrosyl protein when incubated with anti-sheep IgG Ab. (3) Anti•Tyr-P Ab bound to phosphotyrosyl protein electrophoretically transferred to nitrocellulose paper. Little to no cross reactivity was seen with pmol levels of authentic phosphoserine proteins. Peroxidase labeled anti-sheep IgG Ab allowed visualization of proteins containing Tyr-P residues on Western blots at a sensitivity limit of ~50 fmols. Autoradiogram intensities from 32P labeled cell proteins were comparable to immunostains only after base treatment to dephosphorylate Ser-P/Thr-P residues. Immunostains of Ehrlich ascites tumor cell proteins revealed prominent bands of a Mr ~97,000 protein found in membranes and a Mr ~30-35,000 cytosolic protein. Immunological identification of proteins containing Tyr-P may be used to characterize certain cells, and those proteins which are targets for oncogene derived protein tyrosine kinases.

GPO 914-918

Project Description:

Objectives: The role of proteins containing Tyr-P in eucaryotic cellular function is of considerable interest since it was found that a retrovirus gene transforming product was a protein tyrosine kinase. Also, receptors for insulin and several growth factor receptors (epidermal, platelet, and two tumor types) display protein tyrosine kinase activity, presumably as an intrinsic part of their receptor. Four primary goals were set to allow biochemical investigation of the interconverting enzymes and their substrates:

1. Detection and quantification of Tyr-P in proteins.

2. Assay of the enzymes responsible for the phosphorylation of protein tyrosine residues.

3. Assay of the enzyme responsible for the dephosphorylation of protein Tyr-P residues.

4. Production of antibodies which recognize Tyr-P residues in proteins.

Major Findings:

Antibodies produced by sheep immunized with a Tyr-P bovine serum albumin conjugate and affinity purified bind to phosphotyrosyl glutamine synthetase but not to adenylylated glutamine synthetase which suggests that their specificity for Tyr-P is the sole reason for the specific interaction. The antibodies allow the decoration of proteins containing Tyr-P transferred to nitrocellulose after SDS polyacrylamide gel electrophoresis [PAGE] when used in conjunction with peroxidase bound rabbit antibodies which bind sheep immunoglobulins. Extracts from Ehrlich ascites tumor cells show immunoreactive bands with the anti-Tyr-P antibodies. These proteins which are immunoreactive are found in the cell membrane fraction (M_r ~100,000) and in the cytosol (M_r ~35,000) after 20,000 x g centrifugation. Their cellular function is unknown. Further proof that a immunodecorated band contains Tyr-P can be obtained by treating the [^{32}P] radiolabeled bands with base (1 N KOH, NaOH) at 65° for 1 hr followed by autoradiography. The bands which retain radioactivity contain Tyr-P or Ser-P and Thr-P which resist base (often due to having proline adjacent to the modified amino acid). It was found that electrolytic transfer of radiolabeled proteins from SDS gels to a nylon based blotting paper allowed the base treatment to be easily done. Casein modified with cyclic AMP protein kinase lost virtually all of the ^{32}P label after this treatment while the [^{32}P]phosphotyrosyl glutamine synthetase radioactivity remained. This treatment has been applied to ^{32}P labeled proteins of normal and transformed rat kidney fibroblasts. Distinct ^{32}P labeled protein bands are seen in these cell lines under conditions where cell membrane preparations are incubated at 0° on ice with [^{32}P]ATP prior to SDS gel analysis. This condition was chosen since many of the tyrosine kinases can be autophosphorylated on ice. Major base resistant bonds are seen where proteins of M_r ~100-150,000 migrate.

Significance to Biomedical Research and the Program of the Institute:

The control of cell growth and transformation has been related to the phosphorylation of tyrosine residues. Understanding the role and regulation of this activity will be useful to understanding a variety of human diseases at the molecular level.

Proposed Course:

The demonstration that [^{32}P]protein bands on SDS gels which are resistant to base and are immunoreactive with anti•Tyr-P antibodies can be diagnostic for certain transformed cells, or cells containing insulin or epidermal growth factor receptors. Secondly, the isolation and characterization of substrates for the kinase by the use of affinity columns of immobilized anti•Tyr-P antibodies. The role of tyrosine phosphorylation in cell growth and transformation requires the identification of the cellular activities of the substrates.

Publications:

Gentleman, S., Russell, P. Martensen, T.M., and Chader, G.J.: Characteristics of Protein Tyrosine Kinase Activities of Y-79 Retinoblastoma Cells and Retina. Arch. Biochem. and Biophys. 239: 139-136, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00246-04 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Incorporation of Selenium into Bacterial Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Mark X. Sliwkowski, Staff Fellow, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The clostridial glycine reductase complex requires a selenium-containing protein, selenoprotein A, as one of its essential components. This enzyme complex catalyzes the reductive deamination of glycine, which is coupled to the esterification of orthophosphate resulting in the formation of ATP. A unique selenocysteine (SEC)-containing peptide from selenoprotein A has been purified and subjected to automated Edman degradation. The sequence of the peptide is VAL-SEC-THR-ALA-ALA-GLY-ALA-MET-ASP-LEU-GLU-ASN-GLN-LYS. In addition to selenocysteine, the protein also contains two other cysteine residues. The mechanism by which Se is incorporated into a specific residue of the polypeptide chain remains unknown. In order to address this question, antibodies to selenoprotein A have been raised. To increase its antigenicity, selenoprotein A (Mr = 12000) was coupled via its sulfhydryl/selenol group(s) to the amino groups of serum albumin using m-maleimidobenzoyl-N-hydroxysuccinimide ester. Using dot-immuno assays and immunoblotting of SDS-polyacrylamide gels, the synthesis of selenoprotein A by Clostridium sticklandii under various growth conditions was examined. Cells grown under limiting conditions (1 nM Se) have 50-100 times lower amounts of active selenoprotein A and no precursor forms were detected after DEAE-HPLC fractionation of extracts. Active and inactive forms of selenoprotein A (i.e., lacking Se) have been found in preparations from cells grown with normal Se levels (1 μM).

In a collaborative effort with Dr. August Böck from Munich, studies on the structure of a cloned selenoprotein, formate dehdyrogenase, have been initiated. By comparing information obtained by sequencing both the gene and the peptide, the codon and the precursor amino acid will be determined. An alternative amino acid analysis technique, using phenylisothiocyanate, has been introduced to the laboratory. This procedure can be used to quantitate both primary and secondary amines. An added advantage is that selenium amino acids are also easily identified and quantitated.

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Project Description:

Objectives: The emphasis of our research has been on the isolation and characterization of selenium-containing proteins. Several enzymes, from both bacterial and mammalian sources, have been shown to contain covalently bound selenium. This laboratory has been chiefly concerned with those proteins of bacterial origin. Glycine reductase activity in many amino acid-fermenting clostridia is dependent on the presence of selenium in the culture media. One of the components of this complex, selenoprotein A, has been shown to contain a selenocysteine residue, which is required for the formation of an active glycine reductase complex. The goals of this project have been to determine the function of selenium at the molecular level and to establish the mechanism for its specific incorporation into proteins.

Major Findings:

1) The selenocysteine-containing peptide from the glycine reductase complex of C. sticklandii has been purified and sequenced. Cleavage of the ^{75}Se-carboxamidomethylated protein with trypsin, chymotrypsin, or endoproteinase Arg-C, yields a single ^{75}Se peptide. These results are in agreement with earlier studies which indicated a single site of selenium incorporation within the polypeptide structure of the protein. The purification procedure developed for the selenium-containing peptides consists of an initial reverse-phase HPLC step. The tryptic and chymotryptic peptides were then further purified by HPLC cation-exchange chromatography in pH 3 sodium phosphate containing 30% acetonitrile. The column was then eluted with an increasing sodium phosphate gradient. As determined by automated Edman degradation, the sequence of the tryptic peptide is:

VAL-SEC-THR-ALA-ALA-GLY-ALA-MET-ASP-LEU-GLU-ASN-GLN-LYS

All of the ^{75}Se was associated with the second cycle of the Edman degradation. The fact that only background counts were found on the eighth cycle, which contains a methionyl residue, is of particular interest. Thus, in contrast to the selenomethionine-containing proteins which were investigated earlier, this selenocysteine protein contains selenium at a single specific site and requires the element for enzymatic activity.

2) Earlier studies have indicated that selenoprotein A was not very antigenic, probably due to the fact that it is a small protein (M_r = 12000). To increase its antigenicity, selenoprotein A was coupled via its sulfhydryl/selenol group(s) to the amino groups of serum albumin using m-maleimidobenzoyl-N-hydroxysuccinimide ester. Using dot-immunoassays and immunoblotting of SDS-polyacrylamide gels, the synthesis of selenoprotein A by Clostridium sticklandii under various growth conditions was examined. Cells grown under limiting conditions (1 nM Se) have 50-100 times lower amounts of active selenoprotein A and no precursor forms were detected after DEAE-HPLC fraction-

ation of extracts. These data suggest that the presence of Se in the medium controls the synthesis of selenoprotein A. Interestingly, it was discovered many years ago by Dr. Thressa Stadtman that the addition of purified selenoprotein A restored full activity to selenium deficient cultures, suggesting that the B and C components of glycine reductase are still being synthesized.

3) A new pre-column amino acid analysis procedure using phenylisothiocyanate (PITC) has been introduced to the laboratory. Although two other methods of amino acid analysis using o-phthaldialdehyde (OPA) have been used for some time, neither of these methods were satisfactory for determining secondary amines such as proline. Interest in quantifying proline prompted development of this technique. An added advantage of this procedure is that carboxymethylcysteine and carboxymethylselenocysteine are baseline resolved from the other amino acids (eluting between glutamate and ser) by this procedure.

Proposed Course:

The selenium-containing subunit of formate dehydrogenase from E. coli has been cloned by August Böck and colleagues from the Institut Genetik and Mikrobiologie der Universistät München. We have obtained the strain from Dr. Böck and initiated a collaborative project. We have begun purifying the selenium-containing subunit and plan to isolate and sequence the selenopeptide. Dr. Böck's group is now sequencing the gene for this protein. From these results the codon for the putative precursor amino acid for the selenocysteine residue will be determined.

Antibodies to this subunit will also be raised, to assess whether the cloned formate dehydrogenase product appears to be under the same control mechanism as selenoprotein A of glycine reductase from C. sticklandii.

Studies on the synthesis of selenoprotein A will also be continued. We have found that the antibody raised to the C. sticklandii protein cross reacts with the selenoprotein A component from C. sporogenes. C. sporogenes may in fact be a better organism in which to investigate synthesis, since it exhibits good growth on glycine as well as pyruvate and proline. By shifting cultures from one medium to another (and perhaps back again), further insights on the control of the synthesis of this protein and the role of selenium in this process should be gained.

A nucleic acid probe corresponding to the peptide from selenoprotein A which we have isolated and sequenced will be synthesized. This probe will be valuable in determining whether the control of selenoprotein A synthesis is at the transcriptional or translational level. In addition, it would be extremely useful if a cloning project involving selenoprotein A were to be initiated.

Since we will soon have information on the nature of the selenoprotein precursor, both from the protein and DNA level, peptides corresponding to the precursor selenium peptide could be easily synthesized. This peptide could

then be used to characterize the selenium incorporation reaction with respect to the enzyme(s), cofactors, and precursor selenium compounds required.

Acquisition of a cloned selenoprotein would also permit detailed studies regarding the role of selenium in catalysis. A systematic study, involving site-directed mutagenesis, would involve specific substitution of various amino acid analogues for active site residues. By these procedures, insights into the catalytic advantage and essentiality of selenium might be gained.

Publications:

Sliwkowksi, M.X. and Hartmanis, M.G.N.: Simultaneous Single Step Purification of Thiolase and NADP-Dependent 3-Hydroxybutyryl-CoA Dehydrogenase from Clostridium kluyveri. Analytical Biochemistry 141: 344-347 (1984).

Sliwkowski, M.X. and Stadtman, T.C.: Incorporation and Distribution of Selenium into Thiolase from Clostridium kluyveri. J. Biol. Chem. 260: 3140-3144 (1985).

Sliwkowski, M.X. and Levine, R.L.: Labeling of Cysteine-Containing Peptides with 2-Nitro-5-thiobenzoic Acid. Analytical Biochemistry 147: 369-373 (1985).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00247-04 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Proteolysis of Glutamine Synthetase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Jo Ellen Roseman, Guest Worker, Laboratory of Biochemistry, NHLBI

Others: E. R. Stadtman, Chief, Laboratory of Biochemistry, NHLBI
R. L. Levine, Senior Investigator, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Glutamine synthetase and other enzymes can be covalently modified by a variety of mixed function oxidases from bacterial and mammalian sources. It has been suggested that this oxidative modification specifically marks those enzymes for degradation. If so, then there must be proteases which recognize the modified forms and degrade them.

A protease has now been detected and purified from E. coli which specifically degrades oxidized glutamine synthetase. The initial cleavage releases a 3600 MW peptide from the carboxy terminal end of the protein. Native glutamine synthetase is not a substrate.

The protease purification scheme includes ammonium sulfate fractionation, DEAE cellulose chromatography at pH 7.5, hydrophobic chromatography on phenyl Sepharose and HPLC phenyl, and HPLC DEAE ion exchange chromatography at pH 9.0. The purified protease is homogeneous on SDS-polyacrylamide gel electrophoresis.

The properties of this protease distinguish it from proteases previously purified from E. coli. Its molecular weight is 75,000, and it is monomeric. Optimum activity of the protease is at pH 9. Its activity is inhibited by the chelating agents EDTA and o-phenanthroline but not by diisopropylfluorophosphate or other serine protease inhibitors. ATP does not enhance its activity. In addition to degrading oxidized glutamine synthetase, the protease degrades acetylated and carboxymethylated glutamine synthetase as well as insulin and casein but not chromogenic peptide substrates of serine proteases or collagenase. The preferred cleavage sites have been determined using oxidized insulin B chain as substrate.

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Project Description:

Objectives: To elucidate the mechanism of intracellular protein turnover, we have been attempting to isolate from bacteria a system that will degrade normal cell proteins. Studies originally done in 1977 showed that glutamine synthetase (GS) activity is lost in nitrogen-starved bacterial cells prior to loss of GS cross-reacting protein. This led to the hypothesis that a modification which results in inactivation provides a signal to the proteolytic machinery of the cell. The inactivating activity found in bacterial extracts can be mimicked by a model system, consisting of iron, oxygen, and ascorbic acid. Escherichia coli extracts preferentially degrade purified, ascorbate modified GS as compared to native GS. The ascorbate modified GS has been shown to be similar to native GS: it comigrates on native and SDS polyacrylamide gels, has the same fluorescence emission spectrum and shows only subtle differences in its UV absorption spectrum. The ascorbate inactivated GS shows the loss of a single histidine residue per 50,000 molecular weight subunit and the addition of a carbonyl group.

The goal of this project is to purify and characterize a proteolytic activity that preferentially degrades the ascorbate-modified GS over the native, presumably by recognition of the subtle and specific modification imparted by ascorbate treatment.

Methods Employed:

The approach taken was to use a known physiologic substrate and try to find an E. coli protease that will degrade it. By growing cells on radioactive nutrients and allowing them to incorporate the nutrients into protein, we have produced radioactive protein that is unmodified by isotopic labeling techniques.

GS was purified by the zinc precipitation method from an overproducing E. coli K12 strain grown on radioactive amino acids. The purified GS was of high enzymatic specific activity and was uniformly labeled with ^{14}C-amino acids. A portion of this native enzyme was inactivated by the ascorbate model inactivating system.

The radioactive GS is insoluble in trichloroacetic acid, and we developed an assay to measure the production of trichloroacetic acid soluble radioactivity as a result of protease treatment.

Purification of the protease has employed fractional precipitation, conventional as well as HPLC ion exchange and hydrophobic chromatography.

The rates of degradation of other radioactive substrates by the purified protease were measured. Substrates tested included [^{14}C]methyl hemoglobin, [^{14}C]methyl-α-casein, and [^{125}I]insulin, as well as radioactive GS which had been modified either by nitration, acetylation, adenylylation, or urea denaturation/carboxymethylation.

The products of glutamine synthetase or insulin B digestion were isolated using HPLC reverse phase chromatography. Amino acid analysis of the products was performed using the o-phthaldialdehyde precolumn derivitization method.

Major Findings:

Starting from E. coli whole cell extracts, a proteolytic activity has been purified which degrades oxidized GS at least forty times more rapidly than the native. The protease precipitates in a 60-70% ammonium sulfate cut, does not bind to DEAE ion exchanger at pH 7.5, binds to HPLC Phenyl in 1M ammonium sulfate, and binds to HPLC DEAE at pH 9. Following purification using these steps, the protease chromatographs on HPLC gel chromatography as a single protein peak of molecular weight 75,000.

The purified protease is inhibited by aprotinin but not by other serine protease inhibitors. Chelating agents and thiol reagents inhibit the protease, and these effects are not reversible. The alkylating agent ethyleneimine also permanently inactivates the protease. The protease has an alkaline pH optimum and its isoelectric pH is in the neutral range.

Degradation of commonly used radioactive substrates was measured. The protease degrades both casein and insulin but not hemoglobin nor BSA to TCA-soluble products.

The ability of the protease to recognize other modifications of glutamine synthetase was examined. The protease degrades glutamine synthetase in which tyrosine residues are acetylated, as well as glutamine synthetase which is denatured by urea and then carboxymethylated.

The protease degrades all of the modified forms of GS to the same products. Degradation of oxidized insulin B chain by this protease produces 6 products. Information obtained from amino acid analysis of each product and the known insulin B sequence demonstrates that the protease cleaves insulin B at 3 sites:

11 12 13
Leu-Val↓Glu
17 18 19 OOH
Leu-Val↓Cys
15 16 17
Leu-Tyr↓Leu

The purified protease initially cleaves the oxidized glutamine synthetase near the carboxy terminus, yielding 3600 and 46,400 MW products.

Publications:

Roseman, J.E.: Turnover of Glutamine Synthetase: Purification and Characterization of a Protease Which Degrades the Oxidized Form of the Protein. Ph.D. Dissertation, Johns Hopkins University, 1985.

Rivett, A.J., Roseman, J.E., Oliver, C.N., Levine, R.L., and Stadtman, E.R.: Covalent Modification of Proteins by Mixed-Function Oxidation: Recognition by Intracellular Proteases. In Khairalah, E.A, Bond, J.S., and Bird, J.W.C. (eds.): Intracellular Protein Catabolism. New York, Alan R. Liss, 1985, pp. 317-328.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00248-03 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mixed-Function Oxidation of Proteins: Implication in Protein Turnover

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

A. Jennifer Rivett, Visiting Fellow, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mixed-function oxidation of E. coli glutamine synthetase by ascorbate, oxygen, and iron has previously been shown to cause inactivation of the enzyme and enhanced susceptibility to proteolytic attack by a variety of proteases. One of these proteases, a high molecular weight nonlysosomal liver proteinase which does not degrade native glutamine synthetase at neutral pH, has been purified and characterized, and used to study the degradation of the oxidatively modified enzyme. In earlier studies only partial degradation of oxidatively inactivated glutamine synthetase was observed because only a fraction of the subunits had been rendered susceptible to proteolytic attack. Prolonged exposure to the ascorbate system results in more extensive susceptibility to degradation without causing a major conformational change. Study of the time course of exposure to the ascorbate system showed a lag before the increase in susceptibility to proteolysis, during which the enzyme was almost completely inactivated. It has been demonstrated that mixed-function oxidation causes a number of changes in the glutamine synthetase molecule and that these changes do not occur simultaneously. Moreover, different mixed-function oxidation systems may cause different effects on the protein and do not necessarily render the enzyme susceptible to degradation. Several mammalian enzymes have been shown, like glutamine synthetase, to be preferentially degraded after exposure to the ascorbate system but some apparently are not. Comparison of the results with the purified proteinase with those obtained by microinjection of control and oxidized ^{14}C-labeled glutamine synthetase suggest that the increased proteolysis observed *in vitro* likely reflects susceptibility to degradation *in vivo*.

Project Description:

Objectives: Previous studies in this laboratory have suggested that oxidative modification of bacterial glutamine synthetase (GS) marks the enzyme for degradation. The main objective of this study was to investigate the possible role of mixed-function oxidation of proteins in intracellular protein turnover. Specific aims were as follows: (a) to further characterize an intracellular mammalian protease which degrades the oxidized enzyme but not native GS at neutral pH; (b) to examine the effect of mixed-function oxidation of other enzymes on their susceptibility to proteolysis; (c) to determine the basis for the recognition of oxidized GS as a substrate for proteolytic attack; (d) to investigate the mechanism of degradation of oxidized GS.

Methods Employed:

Purified E. coli GS was oxidized by a mixed-function oxidation system composed of ascorbate, oxygen, and iron. The subsequent increased susceptibility to proteolysis was studied by measuring the formation of trichloroacetic acid soluble products after incubation with a purified nonlysosomal liver proteinase.

Major Findings:

(1) Further Characterization of a Liver Alkaline Protease Which Degrades Oxidatively Modified But Not Native GS at Neutral pH. A nonlysosomal alkaline protease which degrades the oxidatively modified form of E. coli glutamine synthetase has been purified to apparent homogeneity from rat and mouse liver acetone powders. Its molecular weight was determined to be 300,000 by Sephacryl S-300 gel filtration but results of further studies using HPLC gel filtration suggest a value of 650,000. Examination of the subunit structure by SDS polyacrylamide gel electrophoresis showed multiple bands of molecular weights between 22,000 and 34,000. The alkaline protease was inhibited by thiol reagents. Phenylmethylsulfonyl fluoride, aprotinin, leupeptin, antipain, and chymostatin partially inhibited the protease. The inhibition by phenylmethylsulfonyl fluoride was prevented by dithiothreitol, and α_1-antitrypsin and soybean trypsin inhibitor did not inhibit. No inhibition was observed with metalloprotease inhibitors. The alkaline protease is active over a broad range of pH with optimum activity for the degradation of oxidized glutamine synthetase around pH 9.0. Its activity is not stimulated by MgATP. A study of the products of insulin B chain degradation demonstrated major cleavage sites of Gln_{13}-Ala_{14}, Leu_{15}-Tyr_{16}, $Cys(SO_3H)_{19}$-Gly_{20}, Gln_4-His_5, and Leu_{17}-Val_{18}. Based on its endopeptidase activity and its inhibitor specificity, the alkaline protease should be classified as a cysteine proteinase. It appears to be distinct from previously described proteinases and is likely involved in nonlysosomal mechanisms of intracellular protein turnover.

(2) Degradation of Oxidized GS. It has now been demonstrated that inactive, oxidized glutamine synthetase preparations used previously were only partially degraded by this proteinase. After oxidative inactivation by

the ascorbate mixed-function oxidation system, some GS subunits were degraded to acid soluble products with no intermediates detected by SDS polyacrylamide gel electrophoresis. The remaining subunits had not become susceptible to proteolytic attack during the limited exposure to the ascorbate system. A study of the time course of the oxidation demonstrated a lag in the enhancment of proteolytic susceptibility. The cysteine proteinase catalyses extensive degradation of oxidatively modified GS to acid soluble products.

(3) Characterization of the Changes Occurring During Mixed-Function Oxidation of Glutamine Synthetase (collaborating with R. L. Levine of this laboratory). Enzymatic activity was lost early in the time course. This oxidative inactivation was accompanied by the loss of one histidine residue per subunit and by a decrease in heat stability of the residual catalytic activity. There was also an increase in carbonyl content, measured by ^{3}H incorporation from sodium borotritide. Longer exposure to the ascorbate system (up to 8 hours) resulted in a continued increase in carbonyl content, loss of a second histidine residue, and a dramatic increase in susceptibility to proteolytic attack. Except for the loss of histidine residues, there were no detectable changes in amino acid composition. Some nonenzymatic cleavage by the ascorbate system was observed, but it did not account for the increase in susceptibility to proteolysis. The glutamine synthetase retained its dodecameric structure throughout the oxidation, except for about 10% which formed aggregates during prolonged exposure. There was no change in sedimentation velocity of the dodecamer, suggesting that there is no major structural alteration in the oxidized enzyme. However, a slight increase in sulfhydryl exposure was detected, and there were differences in spectral changes associated with the removal of metal ions.

(4) Effect of Mixed-Function Oxidation of Other Proteins on Their Susceptibility to Degradation. Several mammalian enzymes which are known to be inactivated by mixed-function oxidation were tested as substrates for the proteinase. Native rabbit muscle enolase and pyruvate kinase were resistant to degradation, but their oxidatively inactivated forms were degraded. Oxidized rabbit muscle phosphoglycerate kinase and creatine kinase were also preferentially degraded. Moreover, trypsin degraded oxidized preparations of all of these enzymes faster than control preparations. Oxidative inactivation of bovine liver superoxide dismutase by hydrogen peroxide caused a slight increase in susceptibility to proteolytic attack, but the enzyme was still relatively resistant to degradation both by the liver proteinase and by trypsin. Although oxidation conditions may not have been optimal for demonstrating enhanced proteolytic susceptibility, the results do indicate that mixed-function oxidation can render mammalian, as well as bacterial, enzymes susceptible to degradation by an intracellular proteinase and by trypsin. However, one example has been found where this is clearly not the case. Oxidized preparations of glucose-6-phosphate dehydrogenase were found to inhibit the degradation of oxidized ^{14}C-labeled glutamine synthetase more than corresponding control preparations, but they were not preferentially degraded (A. J. Rivett, M. E. Wittenberger, and E. R. Stadtman).

(5) Investigation of the Susceptibility of GS to Proteolysis After Mixed-Function Oxidation by Different Mixed-Function Oxidation Systems. No increase in susceptibility of GS to proteolytic attack was observed during oxidation by $FeSO_4$, $FeCl_3$, and oxygen, or by the ascorbate/$FeCl_3$/O_2 system containing EDTA.

(6) Comparison of In Vitro Susceptibility of Native and Oxidized GS to That Observed Following Microinjection (collaborating with J. F. Hare, Department of Biochemistry, University of Oregon). Preliminary results indicate that native ^{14}C-labeled GS is stable after microinjection into hepatoma cells whereas the oxidatively modified enzyme is rapidly degraded within the cells. These results are comparable to in vitro observations with the intracellular proteinase.

Significance to Biomedical Research and the Program of the Institute:

Little is known of the mechanism of regulation of intracellular protein turnover. Mixed-function oxidation can render proteins susceptible to proteolytic attack and may be a mechanism of marking proteins for degradation in vivo. Mixed-function oxidation of proteins has also been implicated in host defense, in oxygen toxicity, and in aging. It is therefore of interest to characterize the increased susceptibility to proteolysis and the other changes which occur in proteins as a result of exposure to mixed-function oxidation systems.

Proposed Course:

Continuation of the above studies (until November 1985).

1. Investigation of the changes in GS during mixed-function oxidation and the basis for recognition by proteases.

2. Investigation of the effects of other mixed-function oxidation systems on the susceptibility of GS to proteolysis.

3. Investigation of the mechanism of degradation of oxidized GS by the cysteine proteinase.

Publications:

Rivett, A.J., Roseman, J.E., Oliver, C.N., Levine, R.L., and Stadtman, E.R.: Covalent Modification of Proteins by Mixed-Function Oxidation: Recognition by Intracellular Proteases. In Khairalah, E.A, Bond, J.S., and Bird, J.W.C. (eds.): Intracellular Protein Catabolism. New York, Alan R. Liss, 1985, pp. 317-328.

Rivett, A.J.: Preferential degradation of the oxidatively modified form of glutamine synthetase by intracellular mamalian proteases. J. Biol. Chem. 260: 300-305, 1985.

Rivett, A.J.: Purification of a liver alkaline protease which degrades the oxidatively modified form of glutamine synthetase: Characterization as a high molecular weight cysteine proteinase. J. Biol. Chem., in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00252-03 LB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of the Mg(II)-ATP-dependent Phosphoprotein Phosphatase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Stewart R. Jurgensen, Staff Fellow, Laboratory of Biochemistry, NHLBI

Others: P. Boon Chock, Chief, Section on Metabolic Regulation, LB, NHLBI

COOPERATING UNITS *(if any)*
Jackie Vandenheede, Katholieke Universitiet te Leuven, Belgium
Susan Taylor, University of California, San Diego, California

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have continued the investigation of the structure, activation, and regulation of the Mg(II)-ATP-dependent phosphoprotein phosphatase. New methods of enzyme purification have been developed utilizing high performance liquid chromatography(HPLC). The enzyme from rabbit skeletal muscle has been purified approximately 17,000 fold to near homogeneity. The enzyme consists of a 38 KDa catalytic subunit and a 31 KDa regulatory subunit, which has historically been referred to as modulator or inhibitor-2. A 62 KDa polypeptide, which previously co-purified with enzyme activity, was separated with the new purification procedure. Densitometric scans of SDS gels indicate a 1:1 stoichiometry of the catalytic and regulatory subunits. This, however, assumes identical staining properties of the two subunits. Measurements based on the activity of the catalytic and regulatory (as an inhibitor) subunits have suggested a higher regulatory to catalytic subunit stoichiometry, approximately 2:1. The enzyme specific activity has been found to exhibit a concentration dependence. This is seen with kinase Fa at maximally activating concentrations and suggests an association/dissociation which alters the specific activity of the enzyme. The time course of phosphatase activation by kinase Fa and Mg(II)-ATP and of phosphate incorporation into the modulator subunit was further investigated. Initially phosphate is rapidly incorporated into the modulator subunit. As phosphatase activation proceeds, there is a decrease in the amount of phosphate incorporated. As the phosphatase activity levels off at a steady-state level of enzyme activation, the amount of phosphate incorporated into the modulator subunit plateaus at a low stoichiometry of approximately 0.1 mole Pi per mole. After activating the phosphatase, Mg(II) chelation with EDTA was used to block the activation reaction and allow measurement of the time course of inactivation. A first-order inactivation was observed with a t1/2 of 13-15 minutes, reflecting an isomerization of active enzyme back to its inactive form. The data for phosphatase inhibition by the type II regulatory subunit of cAMP-dependent protein kinase (see last year's report) was subjected to a computer fitting based on a single binding site assumption. The good fit that was obtained demonstrated that binding of RII to a single site on the phosphatase can account for the results.

Project Description:

Objectives: In this project we seek an understanding of the structure and the regulation of the Mg(II)-ATP-dependent phosphoprotein phosphatase. This enzyme is a major phosphorylase phosphatase in skeletal muscle. It also dephosphorylates other enzymes which are regulated by phosphorylation and dephosphorylation reactions and which function in the glycogen cascade system. Thus, the regulation of the phosphatase is important to the integration of metabolic signals to allow a fine control of this pathway.

Major Findings:

HPLC methods of enzyme purification have been explored and found to be advantageous. The enzyme is quite labile. HPLC TSK-DEAE ion-exchange or TSK-phenyl hydrophobic interaction columns, run at room temperature, result in large losses of enzymatic activity even with rapid elution protocols under anaerobic conditions. However, reducing the temperature to 4°C and loading protein samples in 10% glycerol resulted in substantial improvement in the recovery of enzyme activity. The TSK-DEAE HPLC column allowed separation of a 62 KDa polypeptide which had previously co-purified with enzyme activity and with the 38 and 31 KDa subunits. However, on this column a polypeptide at about 68-70 KDa was poorly separated from enzyme activity. This polypeptide could be separated by hydrophobic interaction chromatography on the TSK-phenyl column.

The highly purified enzyme, consisting of 38 and 31 KDa subunits, exhibited a concentration dependent specific activity. This occurred with kinase F_A at maximally activating concentrations. The enzyme possessed a specific activity at high dilution (~0.1 nM) of approximately 13,000 nmole/min/mg. At higher enzyme concentrations (~6 nM), the specific activity decreased to between 3,000 and 4,000 units per mg. This suggests an association/dissociation which affects the specific activity of the activated enzyme.

The time course of phosphatase activation and of phosphate incorporation into the modulator subunit during enzyme activation has been further investigated. Initially phosphate is rapidly incorporated into the modulator subunit. As phosphatase activation proceeds, there is a decrease in the amount of phosphate incorporated, and a low stoichiometry, steady-state level of incorporation is established as phosphatase activation plateaus at a steady-state level of activity. This data agrees with the proposal for activation, where phosphorylation of the modulator subunit triggers the activation of the catalytic subunit. Conversion of the active to the inactive form occurs by a slower isomerization step subsequent to dephosphorylation. Blocking the activation reaction by chelation of Mg(II) with EDTA has allowed the measurement of a first-order conversion of active enzyme to its inactive form.

The data for phosphatase inhibition by the type II protein kinase regulatory subunit (R_2^{II}) was subjected to a computer fit based on a single binding site assumption. The good fit obtained indicated that R_2^{II} binding to a single site on the phosphatase can explain the inhibition data. The K_D

for the binding interaction was dependent on the kinase F_A concentration indicating an antagonism between the binding of kinase F_A and of R_2^{II} to the phosphatase.

Electrophoresis in non-denaturing polyacrylamide gels was investigated as a means of demonstrating a complex between phosphatase and R_2^{II}. Several non-denaturing systems that were tried failed to give a sharp, defined band for the phosphatase or failed to preserve enzyme activity. A tris-barbitol-phosphate system (4°C, running pH ~8) gave a single major band after staining which correlated with the position of enzyme activity. However, this system did not allow identification of a phosphatase-R_2^{II} complex. The mobility of the phosphatase in this system was low and there was only slight resolution between phosphatase and R_2^{II} so that a complex might not be readily detected.

Proposed Course:

Several techniques may be tried to allow identification of a phosphatase-R_2^{II} complex. Experiments using bifunctional cross-linking reagents will be performed. Analysis on SDS-gels will allow detection of cross-linked complexes. Ultracentrifugation may also allow detection of such a complex.

Recently a protein of M_r ~100,000 has been described which can be isolated from the glycogen particle. This protein forms a complex with the 38 KDa phosphatase catalytic subunit. This complex results in a spontaneously active phosphatase. It will be important to determine if this protein also interacts with the Mg(II)-ATP-dependent phosphatase.

Publications:

Chock, P.B., Shacter, S., Jurgensen, S., and Rhee, S.G.: Cyclic Cascade Systems in Metabolic Regulation. Current Topics in Cellular Regulation 27, Academic Press, Orlando, Florida, 1985.

Vandenheede, J.R, Yang, S.D., Merlevede, W., Jurgensen, S.R., and P.B. Chock: Kinase F_A-Mediated Regulation of Rabbit Skeletal Muscle Protein Phosphatase: Reversible Phosphorylation of the Modulator Subunit. J. Biol. Chem. 260, in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00254-03 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Detection of Protein Tyrosine•Tyrosine-Nucleotide Phosphodiesters

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Todd M. Martensen, Guest Worker, Laboratory of Biochemistry, NHLBI

Others: Mark Sliwkowski, Staff Fellow, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.12	0.1	0.02

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Proteolytic enzyme preparations whose substrate specificity requirements are broad can be utilized to digest proteins containing tyrosine-nucleotide phosphodiester residues yielding free tyrosine O-nucleotide. Pronase and proteinase K digests of [32P,3H] adenylylated glutamine synthetase yielded tyrosine O-AMP which migrated as a single spot on thin layer (silica) chromatography, and chromatographed as a discrete peak on reverse phase high pressure liquid chromatography. Confirmation that the isolated radiolabeled material was a phosphodiester was achieved by snake venom exonuclease treatment whih resulted in the formation of [3H,32P]AMP. These results suggest that protease treatment of proteins containing putative tyrosine O-nucleotide residues will yield the free modified tyrosine which can be isolated by high pressure chromatography prior to treatment with snake venom exonuclease for nucleotide identification.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives: Nucleotide linked to protein through phosphodiester bonds has not been found in normal eucaryotic cells. Linkage of nucleotides to protein tyrosine residues by phosphodiester bonds are present in two proteins of E. coli, adenylylated glutamine synthetase and uridylylated P_{II}, these proteins can be used to develop detection methods. Characterization of putative nucleotide phosphodiester bonds to protein tyrosine residues in eucaryotic cells requires assays to detect this novel posttranslation modification. This modification has been demonstrated to result after poliovirus infection of mammalian cells, but it is not known to occur naturally. The development of sensitive assays to detect these modifications would allow the scope of the modification to be investigated.

Major Findings:

Pronase, a mixture of several proteolytic enzymes, and proteinase K added individually or together to a preparation of carboxymethylated [^{32}P,^{3}H] adenylylated glutamine synthetase degraded the protein as judged by silica TLC of the reaction mixture. Time course analysis showed that a single spot resulted from the incubation which contained both ^{3}H and ^{32}P, and was distinct from P_i, AMP, and adenosine. Further incubation of the digest with leucine aminopeptidase and carboxypeptidase Y caused no change in the migration of the unique radiolabeled spot which was assumed to be tyrosine-O-AMP. The labeled material bound to an anion exchange resin (Dowex-1) as well as to a cation exchange resin (Dowex-50). It did not bind to immobilized boronic acid which adsorbs nucleic acids containing viccinal hydroxyl groups. Chromatography of the digest on reverse phase HPLC showed that > 90% of the radioactivity eluted as discrete but tailing peak well separated from the majority of the A^{210} adsorbing material. Spectral characterization of the peak showed a broad band unlike tyrosine or AMP. Treatment of the isolated material with snake venom exonuclease resulted in a time-dependent change in the migration of the labeled material on TLC to that of AMP. This was taken as evidence for a phosphodiester bond in the isolated material. Previous studies have shown three ways to infer a tyrosine-nucleotide residues in a protein labeled with [$^{14}C/^{32}P$] nucleotide: (1) Treatment with snake venom diesterase will release ^{32}P and ^{14}C into the supernatant after protein is reprecipitated with TCA. (2) Treatment with micrococcal nuclease will release ^{14}C but not ^{32}P into the supernatant after precipitation. The specificity of the nuclease will produce a [^{32}P]Tyr-P residue at the nucleotide ligation site. (3) Base hydrolysis of nucleotide ligated to tyrosine through a 5'-phosphodiester linkage produces Tyr-P in 20% yield (in adenylylated glutamine synthetase). These findings suggest a direct demonstration is now possible.

Significance to Biomedical Research and the Program of the Institute:

The ligation of nucleotide to protein is known to indicate either specific viral infection or enzyme regulation. The discovery of these modifications in eucaryotic cells may uncover unique new species of heretofore unknown covalent protein modifications.

Proposed Course:

Purification of sufficient quantities of Tyr-O-AMP will be prepared to allow a quantitative amino acid analysis to detect the tyrosine content and the lack of any other amino acids. Proteolytic digestion of the uridylylated protein, P_{II}, to isolate [^{32}P] tyrosine O-UMP will be tried. Separation techniques to purify Tyrosine O-nucleotide on DEAE columns using high performance liquid chromatography will be applied to proteolytic digests of TCA precipitated cell extracts.

Publications:

Martensen, T.M.: The Role of Substrate Structure in Recognition and Regulation of Enzymatic Interconversion of Proteins. In S. Shaltiel and P. B. Chock, eds., Current Topics in Cellular Regulation: Covalent Modification 27, (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00255-02 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biosynthesis of 8-hydroxy-5-deazaflavin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

L. Edward DeMoll, III, Staff Fellow, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The biosynthesis of 8-hydroxy-5-deazaflavin, a conspicuous cofactor in methane-producing bacteria, has been investigated in Methanococcus vannielii. Isotopic labeling studies with [U-14C]guanosine show that a form of guanine is used for biosynthesis of part of the deazaflavin molecule. This incorporation involves loss of the 8-carbon of the guanine moiety. Measurement of release of 14C from the 8-position of the [8-14C]guanine and [8-14C]GTP serves as the assay for the enzymic activity which carries out this reaction. The activity responsible for the reaction has been partially purified from crude extracts of M. vannielii.

130

Project Description:

Objectives: Methane-producing bacteria have several coenzymes not found in other organisms. 8-Hydroxy-5-deazaflavin (F_{420}) has been reported in Streptomyces but only in very small amounts. This project concerns the investigation of the biochemistry of the synthesis of F_{420} by Methanococcus vannielii.

Major Findings:

Work has involved investigation into the biosynthesis of 8-hydroxy-5-deazaflavin by the methane-producing bacterium, Methanococcus vannielii. Isotopic labeling studies indicate that a form of guanine is used in the biosynthesis of part of the deazaflavin molecule. This incorporation involves a loss of the 8-carbon of the guanine compound. Consequently, I have been attempting to purify and characterize the enzyme activity responsible for this reaction. The assay for this reaction determines the amount of ^{14}C released from the 8-position of [8-^{14}C]guanine or [8-^{14}C]GTP. Enzymic activities that accomplish this reaction have been found in crude extracts of M. vannielii. Whether or not the crude system merely converts one form of guanine to the other or to another, different form is unknown.

It is possible that this initial step is also carried out in a similar manner in flavin and pterin biosynthesis. The assay I employ could also detect these activities, although it is possible that all reactions are carried out by the same enzyme system. It would be easier to envision one activity responsible for flavin and deazaflavin biosynthesis and another for pterin biosynthesis, because in other systems, pterin biosynthesis involves not merely loss of the 8-carbon of GTP, but an Amadori rearrangement forming a bicyclic pterin precursor.

Both enzymic activities have been purified approximately 6-fold so far. Studies on this fraction indicate that both tested substrates are not being acted upon in the same manner. In other words, a single enzyme is not responsible for the release of the 8-carbon from both GTP and guanine. Both enzymic activities are oxygen sensitive, but this inhibition appears to be reversed by removal of the enzyme preparation to an inert atmosphere. In the presence of O_2, dithiothreitol (DTT) seems to have little protecting effect on the enzymic activities. Both enzymic activities are strongly inhibited by Mn^{++} and slightly inhibited by Ca^{++} (1-2 mM) in 50 mM Tris, pH 7.5, 1 mM DTT. Recent studies indicate that Tris is slightly inhibitory to the reactions as are KCl levels above 0.05 M (in 0.05 M Tris, pH 7.5, 1 mM DTT). Phosphate, pH 7.5, is partially inhibitory only at high ($\geq$.2 M) concentrations in the GTP reaction, but is significantly inhibitory to the guanine reaction even at 0.05 M.

Proposed Course:

Presently I am optimizing purification steps involving DEAE, phenyl sepharose, and gel filtration chromatography. Purification of the enyzme involved in the initial step of deazaflavin biosynthesis will allow characterization of the product, and thereby enable determination of the subsequent reactions

to proceed. Resolution of the initial step in pterin biosynthesis from that in flavin and/or deazaflavin biosynthesis could also ultimately show whether the the methyl pterins of methane bacteria are made in a manner similar to the pterins in other organisms.

Publications

DeMoll, E. and Shive, W.: Replacement of biotin by dethiobiotin in the growth of Lactobacillus plantarium. Applied and Environmental Microbiology, submitted for publication, 1985.

DeMoll, E. and Shive, W.: Interference by dethiobiotin in the Lactobacillus plantarium assay for biotin. Analytical Biochemistry, submitted for publication, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00256-02 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Enzyme Inactivation in Red Cells During Aging

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboratory, and institute affiliation)*

PI: Bong-whan Ahn, Visiting Fellow, Laboratory of Biochemistry, NHLBI

Others: E. R. Stadtman, Chief, Laboratory of Biochemistry, NHLBI
C. N. Oliver, Staff Fellow, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In relation to protein turnover or aging, we have studied enzyme inactivation associated with oxidative modification of enzyme protein. Many enzymes including E. coli glutamine synthetase undergo oxidative inactivation in vitro by a variety of mixed-function oxidation systems generating activated oxygen species. A prominent change on oxidative inactivation is formation of carbonyl protein derivative which can react with various carbonyl reagents. The erythrocyte is a very good model for study of protein degradation because it is no longer active in protein biosynthesis. Erythrocytes are also a good model for aging because they can be easily separated by age using density gradient sedimentation. We have fractionated erythrocytes according to age and measured the carbonyl content of purified enzymes.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives: The primary objective of this project is to investigate the possible physiological role of oxidative modification in biological systems as a function of age. We have undertaken studies to determine whether enzymes which accumulate in human erythrocytes as inactive or less active forms are oxidatively modified and whether the modifications are similar to the modifications generated by oxidation systems in vitro.

Major Findings:

We fractionated human erythrocytes by age using Percoll gradient. Efficacy of the erythrocyte fractionation according to age was assessed by variations in specific activities of the marker enzymes. Specific activities of glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase decrease up to 40 ~ 50% in old cells compared to young cells. The carbonyl content was measured using tritium-labeled sodium borohydride or tritium-labeled phenylhydrazine which we prepared by an exchange reaction from cold phenylhydrazine and tritiated H_2O.

In crude hemolysate, carbonyl protein derivatives increased slightly (up to 20%) as a function of cell age. However, the result was different for purified glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase. When we purified glyceraldehyde-3-phosphate dehydrogenase from cell membranes of various erythrocyte fractions and compared carbonyl content, no significant changes were observed as a function of cell age. When glucose-6-phosphate dehydrogenase was precipitated from hemolystates using polyclonal antibodies prepared in sheep and then isolated from SDS-polyacrylamide gels, there was no significant change in carbonyl content of the isolated enzyme as a function of cell age.

Proposed Course:

We are in the process of preparing antibodies against amino acid homopolymers such as polylysine, polyhistidine, and polyproline which were oxidized in vitro by the ascorbate-Fe^{3+} oxidation system. Using these antibody preparations, we plan to isolate oxidatively modified forms of enzymes from erythrocytes in order to study the modifications which may occur in vivo. And we are trying to isolate the active and inactive forms of glucose-6-phosphate dehydrogenase from erythrocytes using antibody against the enzyme.

Publications:

Oliver, C.N., Ahn, B., Wittenberger, M.E., Levine, R.L., and Stadtman, E.R.: Age-related alterations of enzymes may involve mixed-function oxidation reactions. In Adelman, R.C. and Rothstein, M., eds., Review of Biological Research in Aging, Alan R. Liss, New York, in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00258-01 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Diol Dehydratase and Diol Metabolism in Clostridium glycolicum

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Maris G. N. Hartmanis, Visiting Fellow, Laboratory of Biochemistry, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The metabolic pathway of the fermentation of ethylene glycol and propylene glycol by Clostridium glycolicum was investigated and the levels of the five enzymes involved were determined. Diol dehydratase, the first enzyme in the pathway, was found to be membrane bound and very sensitive to oxygen. The enzyme was subjected to various treatments in order to solubilize it from its matrix. Only limited proteolysis was found to release small amounts of enzyme activity from the membrane. Addition of different compounds known to affect the activity of cobamide coenzyme dependent enzymes had no influence on the activity of diol dehydratase, suggesting a novel reaction mechanism. The enzyme was strongly inhibited by several compounds, among them radical scavengers such as hydroxylamine and hydroxyurea. Preliminary EPR experiments in combination with the inhibition experiments indicate the possible involvement of a radical in the catalytic mechanism.

GPO 914-918

Project Description:

Objectives: Clostridium glycolicum, an anaerobic bacterium, ferments ethylene glycol or propylene glycol to the corresponding alcohols and acids. The organism was originally isolated for the purpose of studying cobamide coenzyme dependent enzymes, especially diol dehydratase, since it was known that this enzyme isolated from other organisms contained a cobamide coenzyme. Further studies, however, revealed extremely low levels of B_{12} in this organism and it was suggested that the diol dehydratase reaction did not involve a cobamide coenzyme. The objectives of this project are to investigate the metabolic pathway of the diol fermentation in C. glycolicum and to purify and characterize diol dehydratase, and also to study the possibly novel reaction mechanism of this enzyme in detail.

Major Findings:

(1) The metabolic pathway of the diol fermentation by C. glycolicum was investigated and the levels of the enzymes involved were determined. The pathway was found to involve five enzymes. Diol dehydratase carries out the initial attack on the growth substrate, ethylene glycol or propylene glycol, and converts it to the corresponding aldehyde. One half of the amount of aldehyde produced is reduced by a NAD-dependent alcohol dehydrogenase to ethanol or propanol, depending on the growth substrate. The rest of the aldehyde is oxidized to acetyl-CoA or propionyl-CoA by a CoA-linked NAD-dependent aldehyde dehydrogenase. Phosphotransacylase and acetate kinase then convert the CoA-esters to the other end product of the fermentation, acetate or propionate. All enzymes in the pathway, except diol dehydratase, were found to be constitutive, stable to exposure to oxygen, and present in the cytosol. Diol dehydratase was found to be extremely oxygen sensitive and strongly associated with the cell membrane. The dehydratase was induced by diols and could not be detected in cells grown on glucose.

(2) Diol dehydratase solubilization experiments with various ionic and non-ionic detergents, butanol, phospholipase A_2, or osmotic shock procedures failed to solubilize any diol dehydratase activity. Limited proteolysis using subtilisin or subtilisin + 1% Tween 80 released up to 10% of the total diol dehydratase activity. The enzyme was found to be specific for ethylene glycol and propylene glycol and needs a reducing agent for maximal activity. The pH-optimum is 8.0 and the enzyme has a K_m = 3 mM for ethylene glycol. Diol dehydratase was strongly inhibited by low concentrations of EDTA, EGTA, o-phenanthroline, hydroxylamine, hydroxyurea, and sulfhydryl reagents.

(3) Addition of adenosylcobalamin or high levels of intrinsic factor, a B_{12}-binding protein, had no influence on the reaction rate. Irradiation with light also did not affect the reaction rate. These results suggest that the catalytic mechanism of diol dehydratase from C. glycolicum does not involve a cobamide coenzyme.

(4) Preliminary EPR experiments with membrane preparations containing diol dehydratase showed a typical radical signal. There was essentially no

signal from membranes prepared from cells cultured on glucose, which lack diol dehydratase activity. The EPR data also showed a correlation between the radical signal and diol dehydratase activity in inhibition experiments with hydroxyurea and p-chloromercuriphenylsulfonic acid.

Proposed Course:

Since diol dehydratase was found to be extremely oxygen sensitive, further solubilization procedures will be conducted under rigorous anaerobic conditions inside the anaerobic laboratory (oxygen level $\leq$ 10 ppm) to obtain sufficient material for further purification and characterization. Solubilization experiments will involve prolonged sonication at various pH-values, treatment with different detergent combinations, and limited proteolysis.

The released enzyme will then be purified by various chromatographic techniques inside the anaerobic laboratory and characterized in more detail. Efforts will be made to stabilize the enzyme in the presence of air, so investigations can be conducted outside the anaerobic laboratory.

The purified diol dehydratase will be subjected to EPR experiments in order to obtain information about the possible radical mechanism.

Publications:

Hartmanis, M.G.N.: Diol Dehydratase and Glycol Metabolism in Clostridium glycolicum. Fed. Proc. 44: 470, 1985.

Hartmanis, M.G.N., and Stadtman, T.C.: Diol Metabolism and Diol Dehydratase in Clostridium glycolicum. Arch. of Biochem. and Biophys., in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00259-01 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Kinetics and Regulation of Biochemical Reactions at the Cell Membrane

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: R. Dean Astumian, Staff Fellow, Laboratory of Biochemistry, NHLBI

Others: P. Boon Chock, Chief, Section on Metabolic Regulation, LB, NHLBI

COOPERATING UNITS *(if any)* T. Y. Tsong, Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland
Ponzy Lu, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. A simple treatment for the description of interfacial reaction dynamics was developed. This theory clearly illuminates those conditions under which one- or two-dimensional diffusion (as opposed to three-dimensional diffusion seen in homogeneous reactions) can be expected to enhance the overall efficiency or reaction rate of a bimolecular reaction. Additionally, it was brought to light that in systems with many reactive sites on a single moiety, apparent kinetic cooperative effects can be expected even without site-site interactions.

2. A theoretical treatment for the interaction of a transmembrane electric field with the enzyme function of membrane bound proteins was formulated. The major effects were shown to be:

a) For a constant (DC) transmembrane potential, the magnitude of the field strength determines the position of the conformational equilibrium.

b) The energy contained in an externally modulated (AC) field can be transduced by an appropriate transmembrane protein and stored as either electrochemical potential or chemical bond energy.

c) An enzyme complex located within a membrane can cause localized modulation of a metabolically generated DC transmembrane potential by the well-timed opening and closing of a channel. This allows for the understanding of the events of energy transduction in terms of classical enzyme mechanisms, where one part of the kinetic cycle occurs at a different electric field strength than the other part.

GPO 914-918

Project Description:

Objectives:

1) To study the role of an interface in determining reaction dynamics as applied to multiple active site enzymes, ligand-receptor interactions at cell surfaces, and to binding between regulatory proteins and specific control sites on polynucleotides.

2) To understand biological energy transduction in terms of the utilization of osmotic potential energy to modulate the local electric field around a membrane bound enzyme.

Major Findings:

I. Theoretical Description of Interfacial Reaction Dynamics. Bimolecular reactions in which one of the reactants is localized at an interface while the other reactant is initially molecularly dispersed in the homogeneous phase can occur by two paths. One involves the direct interaction of a homogeneous reactant with its interfacially localized reaction partner, and the other proceeds by initial adsorption of the homogeneous reactant and subsequent surface diffusion to reaction. A branching method for the treatment of the surface dynamics was developed, whereby the total reaction velocity is given by the rate at which reactants enter into a specified, arbitrary configuration, multiplied by the product of the probabilities of the subsequent individual events necesary for reaction to occur. This treamtent is particularly useful for the analysis of reactions which occur by multiple mechanisms since the initial arbitrary configuration may be chosen as that point on the reaction coordinate past which the paths diverge. Based on our theory, we have found that although rate enhancement as compared to an analogous homogeneous reaction can be anticipated only for very low interfacial concentrations of localized reactant, the two-dimensional surface diffusion mechanism accounts for an appreciable portion of the total reactivity within a wide range of circumstances for ligand-cell surface receptor interactions. It is also found that when many reactive sites are localized on a single continuous surface, the diffusion controlled binding kinetics of ligand to those sites will be nonlinear, possibly giving the impression of cooperative binding, even when there exists no thermodynamic interaction between the sites.

II. Investigation of the Binding Kinetics of Cro Protein to Specific and Non-Specific DNA. Motivated by the above theory, we have undertaken a study of the binding kinetics of Cro protein to a specific operator site in collaboration with Professor Ponzy Lu of the University of Pennsylvania. It was initially anticipated that using the stopped flow method to measure the binding kinetics of Cro protein to both non-specific and specific DNA of different lengths, we could establish the importance of one-dimensional diffusion in facilitating the location of a specific control site on DNA by a regulatory protein. Our choice of an experimental system was predicated on the observation that binding of Cro protein to DNA results in a 70% loss in intrinsic tyrosine fluorescence, thus providing an easily followable parameter. Although,

our preliminary experiments have been done under conditions such that the relaxation time of the rapid binding step was beyond the time resolution power of the stopped-flow method, we have observed a fascinating difference in the fluorescence change of repressor upon binding to specific and non-specific DNA. Apparently, in both cases, a very rapid bimolecular step resulting in a large fluorescence loss, which could not be resolved by stopped flow, was followed by a slower unimolecular step. However, while for binding to specific DNA, the latter reaction displayed additional fluorescence loss, the step following binding to non-specific DNA was observed as a small fluorescence increase. Although obviously additional experimentation is required to establish the significance and correct interpretation of our observations, we have formulated the following working model.

Basically, we hypothesize that the protein approaches the DNA in such a manner that it allows the aromatic tyrosines to interact with the DNA, resulting in a very rapid fluorescence loss. Then, if the protein recognizes that it has successfully located its specific binding site (and it seems reasonable that in fact the tyrosines may be very important in this recognition process), a subsequent conformational change allows the establishment of relatively strong pi-pi interactions and concomitant additional fluorescence loss, thereby holding the protein in place. If on the other hand, the protein is not at the specific site, a conformational change moves the tyrosines slightly away from the DNA causing a small fluorescence increase, and facilitating unidimensional sliding until either the specific site is located, or dissociation occurs.

III. Electric Field Induced Energy Transduction and Electroconformational Coupling. Typically, the only intensive thermodynamic parameters given to specify a chemical system are the temperature and relevant concentrations. Pressure, for example, in the absence of indication to the contrary, is usually assumed to be one atmosphere. We have shown that for membrane proteins, the transmembrane potential plays a large role in determining the proteins activity. The particular reason behind the importance of the electric field for such systems lies in the fact that a modest physiological transmembrane potential of 100 mV across a 50 Å membrane represents an effective field strength of 200,000 V/cm, wihich is a very strong field.

Initially motivated by the experimental observation that an oscillating electric field induces active transport of Rb^+ ion into erythrocytes via the Na^+-K^+ATPase, we developed a theoretical kinetic model to describe transduction of electric field energy. Both computer simulation and analytical solution confirm that our model is competent for energy transduction under an externally modulated field.

We have also shown that by appropriately timed opening and closing of an ion channel near an energy transducing enzyme, a metabolically generated DC field can be locally modulated and converted to electrochemical potential energy or chemical bond energy. We extended this concept to explain experimental results on the electric field stimulated ATP synthesis by the submitochondrial particle F_0F_1-ATPase.

Our model treats the energy coupling event as a field-induced conformational change, thereby allowing for the understanding of energy transduction in terms of classical molecular mechanisms of enzyme action.

Significance to Biomedical Research and the Program of the Institute:

Our primary objective is to gain an understanding of the role of a cell's membrane in governing the function and regulation of transmembrane proteins. Such proteins are typically involved in signal or energy transduction, or in the movement of metabolites from one side to the other. The knowledge gained in this study is vital for efforts directed toward the eventual control of diseases related to improper functioning of bilayer enzymes.

Proposed Course:

1) To further develop our understanding of heterogeneous reaction kinetics as applied to cellular systems, we will undertake an experimental investigation of th dynamics of inactivation of dodecameric glutamine synthetase by methionine sulfoximine. The kinetics are known to be non-linear, and a detailed study is expected to provide experimental verification of some of our theoretical results.

2) To perform further experiments on the Cro repressor-DNA system designed to clarify our concepts concerning the role of pi-pi interaction and one-dimensional diffusion.

3) To further develop our models for electric field mediated energy coupling with respect to understanding factors governing efficiency and rate of energy transduction. Additionally, the role of stochastic noise and localized "small system" dynamics will be investigated.

4) To carry out experiments designed to test the in vivo applicability of our energy transduction mechanism. In particular, we intend to attempt to demonstrate that an externally modulated electric field is competent to cause synthesis of ATP. Also, we want to show whether nucleotide binding to the F_1 subunit controls opening and closing of the F_0 subunit as predicted by our model for ATP synthesis by the mitochondrial F_0F_1-ATPase.

Publications:

Astumian, R.D. and Chock, P.B.: Interfacial Reaction Dynamics. J. Phys. Chem. 89: in press, 1985.

Tsong, T.Y. and Astumian, R.D.: Absorption and Conversion of Electric Field Energy by Membrane Bound ATPases. J. Electroanalytical Chem. Section on Bioelectrochem. and Bioenergetics, in press, 1985.

ANNUAL REPORT OF THE CARDIOLOGY BRANCH
National Heart, Lung, and Blood Institute
October 1, 1984 through September 30, 1985

The experimental interests of the Cardiology Branch focus on 1) elucidating the mechanisms responsible for dynamic alterations in coronary vascular resistance; 2) defining the pathophysiology and treatment of coronary artery disease, angina pectoris, and hypertrophic cardiomyopathy; 3) evaluating and treating valvular heart disease; and 4) exploring the etiologies and treatment of dilated cardiomyopathy.

DYNAMIC CORONARY VASOCONSTRICTION AS A CAUSE OF MYOCARDIAL ISCHEMIA

Traditionally, angina pectoris has been considered to result from a fixed stenosis limiting blood flow to the myocardium. As myocardial oxygen demands increase in response to stress, the stenosis attenuates the increase in flow that normally occurs, causing myocardial oxygen demands to exceed myocardial oxygen supply; ischemia, and ultimately anginal pain result. The recognition of vasospastic angina (Prinzmetal, or variant angina) focused attention on the fact that intrinsic vasconstrictor tone of coronary vessels can alter sufficiently to cause myocardial ischemia. However, the concept of vasospastic angina was limited, being conventionally understood as angina precipitated at rest by a profound decrease in coronary flow due to spasm-induced total or near-total occlusion of a large epicardial coronary artery. Over the past two years we have explored the possibility that dynamic coronary vasoconstriction may not only involve large epicardial coronary vessels, but also the small coronary arteries or coronary arterioles, which are responsible for autoregulatory changes in coronary vascular resistance.

Ischemia caused by small coronary artery vasoconstriction in pts with atypical angina: We previously demonstrated that about two-thirds of patients with angina-like pain but normal large coronary arteries had inadequate increases in coronary flow and decreases in coronary resistance in response to pacing-induced stress, an abnormality exacerbated by ergonovine. We concluded that the small coronary arteries of these pts have a reduced capacity to vasodilate in response to increases in myocardial O_2 demand that could result in myocardial ischemia, and that drugs (or endogenous compounds) with vasoconstrictor potential can further compromise vasodilator reserve. We confirmed the hypothesis that these pts were experiencing myocardial ischemia by demonstrating diminished myocardial lactate consumption, increased LV end-diastolic pressure, and abnormalities in LV contraction during pacing.

This year we sought to determine whether the reduced vasodilator reserve occurred only in response to metabolic stimuli (pacing-induced increase in myocardial oxygen demand), or was due to a diminished absolute capacity of the coronary vessels to dilate. To assess this we administered the potent coronary arteriolar vasodilator, dipyridamole. We found that pts with impaired vasodilator reserve in response to increased myocardial oxygen demands also exhibited an abnormally low response to dipyridamole, suggesting that the absolute maximal vasodilator capacity of the coronary vessels is reduced. Thus, our data are

consistent with the hypothesis that some pts with angina-like pain and angiographically normal-appearing large coronary arteries exhibit dynamic abnormalities of the small coronary arteries that restrict the maximal vasodilator capacity of the coronary vessels. This can result in an inadequate vasodilator response to increaes in myocardial O_2 demand, or to actual coronary constriction causing angina pectoris, hemodynamic abnormalities, and impairment of LV systolic and diastolic function. Moreover, one-half of the pts with abnormal vasodilator reserve experienced severe angina pectoris during dipyridamole infusion, despite an increase in total flow. These results are compatible with the concept that potent arteriolar dilators can lead to an intramural coronary steal, which suggests that the flow limitation of these patients is due to narrowing of the small pre-arteriolar coronary arteries, rather than the arterioles per se.

Small coronary artery constriction and myocardial ischemia caused by endogenously present neuropeptides and vasoactive amines: We previously demonstrated that endogenous vasoactive substances, such as neuropeptide tyrosine (NPY) and vasopressin, can cause severe small vessel coronary vasoconstriction, such that the resulting increases in coronary vascular resistance can be sufficiently profound so as to override autoregulatory influences, causing both ischemia and myocardial dysfunction. This past year we completed our studies in which we attempted to elucidate the mechanism by which NPY causes coronary vasoconstriction in dogs. Specifically, by studying the dose response curve of NPY before and after the infusion of verapamil, we sought to determine whether the vasoconstriction was mediated by calcium entry through the slow calcium channel. NPY caused the same degree of vasoconstriction with or without verapamil, indicating that NPY-induced vasoconstriction does not require the transport of extracellular calcium through verapamil sensitive channels.

Influence of alterations in blood lipids and coronary atherosclerosis on coronary vascular reactivity: In this study we examined the question of whether an atherosclerotic diet administered to pigs, resulting in elevated LDL cholesterol, alters vascular responsiveness to vasoconstrictor agents. Pigs were fed an atherosclerotic diet and studied 1) in several weeks when serum LDL cholesterol levels were elevated but atherosclerosis had not occurred, and 2) in several months when atherosclerotic lesions were present. A marked impairment of the coronary vasodilator response to histamine was evident in pigs administered the atherosclerotic diet. There was no abnormality in the reactive hyperemic response to coronary occlusion, indicating that lumen obstruction was not the basis for the impaired histamine response. Indeed, the pigs fed the atherosclerotic had only microscopically evident atherosclerosis. Hence, these data suggest that increased lipid levels can alter the interaction between histamine and the vessel wall.

NEW APPROACHES TO THE TREATMENT OF REFRACTORY ANGINA PECTORIS

Myocardial neovascularization with angiogenic factors: One of the major problems in cardiology today is how to more effectively treat those individuals with CAD who have proven refractory to conventional therapy, including antianginal drugs and coronary bypass surgery. One potential approach we are currently testing is implantation of the internal mammary artery (IMA) into ischemic regions of the left ventricle. This operation has been applied to pts in the past (Vineberg operation) but the total blood flow the IMA was found to be capable of delivering generally was insufficient to importantly influence

clinical symptoms. A potent factor potentiating the growth of blood vessels (angiogenic growth factor) has been derived from the greater momentum of the cat. Heparin is also believed to be an angiogenic factor. We have therefore initiated studies to assess the ability of angiogenic growth factor, as well as heparin, to potentiate the growth of vascular anastamoses derived from IMA's implanted in chronically ischemic myocardium in a canine model. We are implanting IMA grafts into the anterior wall of foxhounds, which are randomly assigned to receive continous administration into the IMA of either angiogenesis factor, heparin, or normal saline. The area of ventricle into which the IMA graft is placed will be rendered ischemic over a 2-4 week period by positioning Ameroid constrictors around the LAD coronary artery and the first marginal branch of the circumflex coronary artery. Animals are being studied 8 weeks postoperatively to determine "ischemic" myocardial flow at baseline and during maximal vasodilator stimulation, gross anatomic distribution of vascular anastamoses, and histologic density of coronary arteries within ischemic myocardium.

HYPERTROPHIC CARDIOMYOPATHY

Role of the small intramural coronary arteries as a cause of myocardial ischemia: Many patients with hypertrophic cardiomyopathy (HCM) manifest signs and symptoms of myocardial ischemia and dysfunction. Studies in our laboratory demonstrated that at autopsy, approximately 15% of pts had transmural myocardial infarction without evidence of large vessel coronary disease. To determine a possible cause of these findings, histologic analysis of myocardium was obtained at necropsy in 48 pts with HCM without atherosclerosis of the large coronary arteries. Over 80% of these pts had numerous abnormal intramural coronary arteries (IMCA) which were most common in the ventricular septum, but also in the anterior and posterior free walls. IMCA's had markedly thickened intima or media, and many evidenced severe luminal narrowing; IMCA's were identified frequently in prominent areas of replacement fibrosis. Hence, morphologically abnormal intramyocardial arteries with markedly thickened walls and narrowed lumen are present in most pts with HCM studied at necropsy, suggesting a role of small vessel disease in the pathophysiology of HCM.

We are performing parallel studies in the catheterization laboratory to determine whether there are abnormal coronary flow responses in pts with HCM. Last year we found that while flow increased initially with pacing, in a substantial proportion of pts flow actually fell with, or shortly after, onset of chest pain. This abnormality was accompanied by an elevation of LVEDP and abnormal lactate metabolism. We then hypothesized that if maximal vasodilation occurred with relatively low myocardial metabolic stress, vasodilator reserve would have been exhausted, thereby making it difficult or impossible for the coronary system to counteract vasoconstrictor stimuli. This hypothesis was substantiated when pts who achieved maximal vasodilator reserve at relatively low paced heart rate demonstrated a decrease in peak flow with the infusion of ergonovine. Abnormal sensitivity to vasoconstrictor stimuli may explain the variation in anginal threshold many HCM pts experience. Additional studies this year demonstrated that symptomatic pts without obstruction to left ventricular outflow develop the onset of ischemia at low coronary flows, suggesting a limited peak flow capacity. This further suggests a possible important role of small coronary artery obstruction as a cause of myocardial ischemia in pts with HCM.

Another observation that might relate to the importance of small vessel disease in producing myocardial ischemia and necrosis in HCM, is our finding that some HCM pts develop severe progressive functional limitation in association with findings suggestive of the development of progessive myocardial scarring. Thus 11 pts with severe functional limitation with only mild localized LVH (and no obstruction to LV outflow) were identified who had long-term echocardiographic follow-up. As their symptoms deteriorated, progressive septal thinning and LV cavity enlargement occurred. Radionuclide angiography demonstrated impared LV systolic and diastolic function in most of these pts. Hence, 1) severe cardiac symptoms may occur in pts with nonobstructive HCM who have only mild localized LVH and 2) the presence of progressive wall thinning and cavity enlargement associated with depressed systolic function suggests that extensive myocardial scarring, possibly due to ischemia caused by abnormal small coronary arteries, is responsible for their functional deterioration.

To further explore the possible mechanisms responsible for the abnormal intramyocardial coronary arteries in pts with HCM, we examined the hypothesis that thrombogenic factors may contribute to lumen narrowing of the IMCA's by studying intrinsic fibrinolytic activity (FA) in 28 consecutive pts with HCM. FA was assessed by measuring tissue plasminogen activator (tPA), inhibitor of tissue plasminogen activator (IPA), and alpha 2- antiplasmin. Fibrinolytic activity was impaired (decreased tPA and/or increased IPA) in 82% of HCM pts. While abnormalities in FA did not parallel other indices of disease severity, we plan long-term clinical follow-up to determine whether HCM pts with impaired FA are at greater risk for intravascular thrombus formation, resulting in ischemia, LV dysfunction, or sudden death.

Does true "obstruction" to LV outflow exist in patients with HCM?: A major controversy persists as to whether true outflow obstruction exists in pts with HCM and subaortic gradients. This question is of critical importance in terms of understanding the basic pathophysiologic mechanisms present in this disease, as well as the potential role of operation, since the surgical approach to HCM is designed only to eliminate or reduce the degree of obstruction. Last year we performed a pulsed Doppler echocardiography study on the patterns of LV emptying in pts with and pts without gradients and HCM. That study demonstrated that over 40% of forward flow occurred after mitral-septal contact (an event marking the onset of gradient and elevated LV pressures). In another study, in which LV pressure-volume loops were determined in HCM pts, we found that over 60% of total LV stroke volume was ejected after onset of the LV outflow tract gradient. These findings provided evidence against the concept that ejection is completed at gradient onset, and that the gradient is merely due to LV cavity obliteration. This year LV ejection dynamics in HCM were compared with those of pts with valvular aortic stenosis, since it has been contended that the very high ejection fraction seen in many pts with HCM is not only a reflection of the early complete emptying of the LV (refuted by the above studies), but also indicates that no physiologically important obstruction to LV outflow occurs. We found that a significant percentage of AS pts manifested supranormal ejection fraction, indicating that such a finding does not exclude significant LV outflow obstruction. In another study flow characteristics were determined in 8 pts intraoperatively who were undergoing surgery for relief of obstruction. A flow probe affixed to the ascending aorta assessed forward flow before and after septal myectomy or mitral valve replacement. Before surgery, and in the presence of a subaortic

gradient, aortic flow persisted through the entire systolic ejection period; almost 60% of flow was ejected after gradient onset, but at gradient onset flow deceleration was abrupt. Ejection time was prolonged. After surgery, with abolition or marked reduction of the gradient, abrupt flow deceleration was no longer present and ejection time was reduced.

We also determined the effects of surgical relief of LV outflow obstruction on coronary flow and myocardial metabolism. We found that surgical relief of LV outflow gradient reduced resting and pacing-induced coronary flow and myocardial oxygen consumption, lowered LVEDP post pacing, and improved anginal threshold as well as the metabolic evidence of ischemia. Thus, all of these studies provide substantial support for the concept that LV outflow gradients reflect a mechanical impediment to LV outflow, and result in important physiologic effects on LV myocardial flow, oxygen demands, and ischemic threshold.

Diastolic dysfunction in HCM and its relation to the magnitude of LVH: The pathogenesis of diastolic abnormalities in HCM is unknown, although it is commonly believed that they result primarily as a consequence of marked LVH. We questioned this assumption, however, since we have previously shown that verapamil can acutely improve diastolic abnormalities in HCM pts. To further investigate the relation between disorders in diastolic function and LVH in HCM, we studied by 2-D echocardiography 52 pts with HCM with different degrees of LVH. Although the pts with the most marked hypertrophy, in general, had the worst impairment of diastolic function, diastolic abnormalities were also found in pts whose LV was only mildly hypertrophied; regional LV wall analysis demonstrated that even nonhypertrophied LV segments exhibited diastolic abnormalities. These findings suggest that diastolic abnormalities in HCM are not merely a consequence of the degree of LV hypertrophy; based on our verapamil data they may be partly due to a disorder in calcium fluxes.

Mechanisms responsible for the functional improvement in HCM pts by amiodarone: Last year we demonstrated that high dose oral amiodarone (1200-1600 mg/day) significantly improves exercise capacity and symptoms in many pts with HCM and refractory symptoms. This year we evaluated the chronic effects of amiodarone in the same patient group while on lower dose maintenance therapy (400-600 mg/day for 2 months). We found that exercise capacity improved significantly during chronic oral therapy, and we now use this drug when other approaches to symptomatic control fail. We also have investigated the mechanisms whereby amiodarone produces its beneficial effects. We found that chronic administration of the drug improved the rate and magnitude of rapid diastolic filling. Moreover, the presence or absence of improved diastolic function correlated with the presence or absence of improved symptoms. Thus, improved diastolic function appears to be an important mechanism contributing to the clinical efficacy of amiodarone therapy in HCM.

CORONARY ARTERY DISEASE

Lasers in the treatment of cardiovascular disease: For the past 3 years a joint effort by the Cardiology and Cardiac Surgery Branches has been dedicated to the exploration of the use of laser technology in the treatment of cardiovascular disease. Our goal is to develop a laser energy source and delivery system that can be used to open obstructing atherosclerotic lesions in the coronary system by techniques that can be employed in the operating room and, ultimately, in the catheterization laboratory. This will be a long-term effort, and will require a multidisciplinary approach. To achieve this goa-

we are conducting studies to develop the three major components that will constitute the laser system. We are: 1) assessing the relative efficacy of different laser energy sources, 2) evaluating small catheter systems that will enable us to visualize the lesion to be lased as well as to deliver the energy source, 3) working with different groups to identify a flexible wave-guide fiber that can be coupled to the laser source and requisite energy levels to ablate the target lesions. During this past year, several studies have been completed.

First, we have defined patterns of tissue injury and ablative efficacy associated with different laser sources. In these experiments, laser energy was delivered to fresh human cadaver coronary arteries and gross morphologic, light microscopic, and thermal characteristics of the different laser sources were evaluated. The latter were explored by using infra-red surface thermography and fast reactive thermocouples applied to the adventitia. Lasers evaluated were continuous Argon, CO2, Nd-YAG, and ultraviolet (excimer -XeCl, KrF). Both short pulse CO2 and excimer lasers resulted in minimal thermal tissue injury associated with excellent ablative efficacy. In contrast, the Argon laser, which is the dominant laser being used in laboratories exploring the potential role of lasers in the treatment of CAD, was highly unsatisfactory. The Argon laser produced explosive vaporization of tissue water with the development of high surface endothelial temperatures, and a broad area of thermal diffusion with histologic thermal injury evident around the lased target. Our studies indicate that the Argon laser is nonselective for plaque, imprecise, associated with severe thermal injury, and results in variable ablative effects that may lead to vessel wall perforation. In contrast, the excimer and CO2 lasers caused highly localized tissue effects, sharp symmetric craters without thermal injury, and minimal adventitial temperature changes.

Other studies were performed to determine whether specific laser source wavelengths are selectively absorbed by atheroma relative to normal intima. If so, it would allow more selective and therefore safer application of lasers to atherosclerotic lesions. We performed spectroscopic analyses of normal and diseased portions of human aorta; atheroma was of variable composition (fatty, fibrous, calcified). The atheromatous lesions demonstrated no preferential absorption at any wave-length (270-2500 nm) compared with normal intima; however, both normal and diseased intima intensely absorbed the mid-ultraviolet and the far infrared regions. Thus, although there is no intrinsic chromophore that will consistently differentiate atheromatous from normal tissue, our results suggest that the excimer (ultraviolet) and CO2 (infrared) lasers are theoretically best suited for plaque ablation, as their wavelengths might provide the best prospect for highly localized ablative effect while minimizing thermal damage to surrounding vessels wall. Experiments have also been completed to develop the technology to directly visualize intravascular anatomy, using ultrathin fiberoptic angioscopes in 5 different animal species and in human cadaver preparations. We developed a system that permits visualization of the carotid and femoral arteries of living animals with circulation intact, and have begun intraoperative video recordings of the proximal coronary artery anatomy in pts undergoing aortic valve replacement or CABG.

LV dysfunction at rest: role of myocardial ischemia and reversibility by CABG: Successful coronary artery bypass surgery (CABG) improves exercise-induced ischemia and LV dysfunction in pts with CAD, but it has been assumed that resting dysfunction usually indicates irreversible disease due to prior myocardial infarction. We studied 28 CAD pts without previous infarction by radionuclide angiography before and 6 mos after CABG. LV ejection fraction (EF) at rest increased (55±9 to 60±8%, $p<.005$), with 20 of 28 pts manifesting an increase compared to preoperative values. Quantitative regional LV analysis indicated that improved EF at rest after CABG occurred in those regions developing ischemia during exercise before CABG. Thus, many CAD pts appear to have subclinical LV ischemia and secondary LV dysfunction at rest, and that these abnormalities are reversible after restoration of coronary flow.

Enhanced regional LV function in non-revascularized myocardium after distant coronary bypass: To determine whether CAB can, through collaterals, improve function in distant LV regions not revascularized (because of nonbypassable arteries or occluded grafts), pts with multivessel CAD were studied by RNA before and 6 mos after CABG. Exercise-induced wall motion abnormalities present preoperatively improved postoperatively and regional EF increased (from 49 to 65%, $p<.001$) in regions that were not revascularized in 10 of the 11 pts who had only some of the stenosed vessels bypassed. Improved collateral perfusion in these regions were demonstrated in 8/10 pts. Hence, pts with a nonbypassable coronary artery may still benefit from CABG if the jeopardized myocardium is perfused by collateral vessels supplied by a stenosed artery amenable to CABG.

DILATED CARDIOMYOPATHY

Limited vasodilator reserve in pts with DCM: In the majority of pts with DCM, the etiology is unknown. However, many DCM pts complain of anginal-type pain despite angiographically normal coronary arteries. To examine the hypothesis that abnormalities of coronary blood flow exist in DCM that are responsible for the ischemic symptoms, and possibly for the progression in LV dysfunction, 20 pts with dilated cardiomyopathy and normal epicardial coronary arteries, 8 of whom had frequent chest pain, underwent measurement of great cardiac vein flow and myocardial metabolism at rest and during pacing to a heart rate of 150. After administration of ergonovine, all 8 patients with the history of chest pain experienced their typical chest pain. Compared to patients without chest pain, their coronary flow was lower and coronary resistance higher with significant widening of the AVO_2 difference suggestive of myocardial ischemia. Additionally, the increase in left ventricular filling pressures was higher in this group. There was no significant change in EKG or epicardial coronary luminal diameter by angiography. Administration of dipyridamole 0.5 to 0.75 mg intravenously to a subgroup of these patients suggested impairment in transmural coronary flow reserve compared to patients without chest pain after ergonovine administration. Thus, patients with dilated cardiomyopathy and chest pain by history may have limited coronary vasodilatory reserve, especially after vasoconstrictor stimulus. Future studies are in progress that may determine whether these abnormalities contribute to the myocardial damage in DCM.

Therapy in DCM: The Cardiology Branch is collaborating with the Medical Intensive Care Unit in a study designed to define the natural history of DCM and the effects of anti-inflammatory treatment. In this randomized study

the effects of standard medical treatment will be compared with those of standard medical treatment plus anti-inflammatory therapy (prednisone and, if not effective, cytoxin). End points are changes in functional status, myocardial histology and function, and survival. To date, 97 pts have been screened and 60 pts randomized: on biopsy 37 had reactive myocarditis and 23 nonreactive cardiomyopathy. There have been 5 deaths to date. We have calculated that the study can be terminated after 60 pts have been randomized to the reactive myocarditis group. It is hoped that this study will enable us to 1) identify pts at high risk of developing progressive LV dysfunction, 2) define some of the underlying causes of the DCM, and 3) determine whether or not anti-inflammatory therapy is effective in controlling the progression of the disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-01672-09-CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Maintenance of a computerized clinical data bank for cardiology patients

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Douglas R. Rosing, M.D.	Head, Cardiac Catheterization Lab.	CB, NHLBI
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Gail Greenberg	Computer Technician	CB, NHLBI
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Larry Martin	Systems Analyst	DMB, DCRT
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COOPERATING UNITS *(if any)*
Data Management Branch (DMB), DCRT
Surgery Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	.1	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A computerized data bank has been established for cardiology patients in order to provide easy access to patient data for clinical and investigative purposes. Included in the data base is information from both outpatient and inpatient visits, as well as the identification of procedures and diagnoses generated at other institutions. The data includes symptom description; x-ray; ECG; catheterization; radionuclide; exercise stress test; ambulatory monitor and echocardiogram results; listing of current medications; and disposition of the patient. In addition, with the assistance of the "MIS" system, a printout of the catheterization results is generated for the medical records. Data accumulation was begun on January 1, 1978, and current and retroactive information has been entered on all of our patients. The data base of the Cardiology Branch and that of the NHLBI Surgical Branch were merged into one system, thus facilitating access to data on all patients with cardiovascular disease. Queries of the data base for clinical and research purposes have averaged about 20 per month. A summary of available "pertinent" clinical data is now obtained for each outpatient visit to the Surgery Clinic in order to make chart reviews easier at these visits.

At the present time, there are 8,100 patients entered into the data base. Of these patients, approximately 5,000 have undergone some cardiac operation.

150

Project Description:

A computerized data bank has been established for cardiology patients in order to provide easy access to patient data for clinical and investigative purposes. Included in the data base is information from both outpatient and inpatient visits, as well as the identification of procedures and diagnoses generated at other institutions. The data includes symptom description; x-ray; ECG; catheterization; radionuclide; exercise stress test; ambulatory monitor and echocardiogram results; listing of current medications; and disposition of the patient. In addition, with the assistance of the "MIS" system, a printout of the catheterization results is generated for the medical records. Data accumulation was begun on January 1, 1978, and current and retroactive information has been entered on all of our patients. The data base of the Cardiology Branch and that of the NHLBI Surgical Branch were merged into one system, thus facilitating access to data on all patients with cardiovascular disease. Queries of the data base for clinical and research purposes have averaged about 20 per month. A summary of available "pertinent" clinical data is now obtained for each outpatient visit to the Surgery Clinic in order to make chart reviews easier at these visits.

At the present time, there are 8,100 patients entered into the data base. Of these patients, approximately 5,000 have undergone some cardiac operation.

Overt the past year, the surgical data base has been revised so that it is more "user friendly". This has been accomplished by writing software for creating subfiles which can then be statistically analyzed (SAS program). These functions can be performed by our computer technicians without the previously needed input from a systems analyst. Similar modification of the cardiology data base could also take place, if it were felt it would be useful.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-01761-07-CB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Three year follow-up of patients undergoing PTCA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Richard O. Cannon, M.D.	Senior Investigator	CB, NHLBI
Rita M. Watson, M.D.	Sr. Medical Staff Fellow	CB, NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB, NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Rita Mincemoyer	Cath Lab Nurse	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Although it has been observed that patients describe marked symptomatic benefit and improved exercise capacity 2-4 years after the performance of percutaneous transluminal coronary angioplasty (PTCA), little data is available documenting anatomic and functional results during long-term follow-up. Thus, 46 of the first 53 patients who had a successful procedure, and had not had clinical or anatomical evidence of restenosis in the first 9 months after the procedure, were reevaluated at an average of 37 months after their last successful procedure. At follow-up patients underwent cardiac catheterization, treadmill exercise testing and radionuclide angiography at rest and with exercise.

The percent diameter narrowings pre-PTCA was 66±13% and improved to 30±13% immediately after the procedure. It was 26±16% six months later and 19±13% at late follow-up. Each successive measurement was statistically significantly less than the narrowing found at the previous study. Treadmill exercise time was 9.8±4.4 minutes pre-PTCA and improved to 18.3±4.5, 20.3±4.6, and 18.2±4.5 minutes at the three respective post-PTCA studies. No significant change occurred in rest LV ejection fraction (EF) after PTCA, but exercise LVED, which had fallen by 4±6% pre-PTCA (compared to rest) rose after PTCA by 7±7, 6±7, and 4±7% respectively. Twenty-two patients showed a greater than 10% decrease in the amount of narrowing at the angioplasty site between the six month and three year studies. Six patients developed new significant stenoses at nondilated sites during follow-up. These results indicate that the short-term anatomic and functional success after PTCA is maintained for at least three years, even though disease occasionally progresses at other sites.

Project Description:

Although it has been observed that patients describe marked symptomatic benefit and improved exercise capacity 2-4 years after the performance of percutaneous transluminal coronary angioplasty (PTCA), little data is available documenting anatomic and functional results during long-term follow-up. Thus, 46 of the first 53 patients who had a successful procedure, and had not had clinical or anatomical evidence of restenosis in the first 9 months after the procedure, were reevaluated at an average of 37 (range=27.5-45.5) months after their last successful procedure. Forty-one patients had single vessel disease and 5 had multiple vessel disease. At follow-up patients underwent cardiac catheterization, treadmill exercise testing and radionuclide angiography at rest and with exercise.

The percent diameter narrowings pre-PTCVA was 66±13% and improved to 30±13% immediately after the procedure. It was 26±16% six months later and 19±13% at late follow-up. Each successive measurement was statistically significantly less than the narrowing found at the previous study. Treadmill exercise time was 9.8±4.4 minutes pre-PTCA and improved to 18.3±4.5, 20.3±4.6, and 18.2±4.5 minutes at the three respective post-PTCA studies. These three values are statistically longer than pre-PTCA but not different from each other. No change occurred in rest LV ejection fraction (EF) after PTCA, but exercise LVEF, which had fallen by 4±6% pre-PTCA (relative to the value at rest), rose after PTCA by 7±7, 6±7, and 4±7% respectively. The post-PTCA values are all statistically similar and significantly different from the pre-PTCA testing. According to the Cnadian Heart Association functional classification, before PTCA 1 patient was asymptomatic, 15 were functional class (FC) II, 24=FC III, and 6=FC IV. At long-term follow-up 25 patients were asymptomatic, 10=FC I, 7=FC II, and 4 were still FC III. Twenty-two patients showed a greater than 10% decrease in the amount of narrowing at the angioplasty site between the six month and three year studies. Six patients developed new significant stenoses at nondilated sites during follow-up and two of these patients are still FC III. These results indicate that the short-term anatomic and functional success after PTCA is maintained for at least three years, even though disease occasionally progresses at other sites.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04067-02-CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Detrimental effect of ergonovine in hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard O. Cannon, III, M.D.	Senior Investigator	CB, NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Patients with hypertrophic cardiomyopathy (HCM) frequently experience chest pain that occurs with variable threshold of onset and is often prolonged in duration. The study was designed to evaluate the effect of a pharmacologic vasoconstrictor agent, ergonovine, on the coronary vasculature of patients with HCM. Twenty-three patients with HCM and a history of angina pectoris despite normal epicardial coronary arteries underwent a study of coronary flow, myocardial mechanics, and metabolism. During pacing to an average heart rate of 133, 18 of the 23 patients experienced their typical chest pain. During pacing after the administration of ergonovine, 21 of 23 patients experienced chest pain. Despite a significantly higher blood pressure, the coronary blood flow at an average pacing rate of 138 beats/min was significantly lower than during pacing alone to a similar heart rate. There was no epicardial coronary artery narrowing during a repeat coronary arteriography. Thus, peak coronary flow decreases with ergonovine in patients with HCM, probably due to vasoconstriction of a maximally dilated microvascular bed, or vasoconstriction of prearteriolar small coronary arteries. Small vessel coronary vasoconstriction may explain many of the atypical features of angina pectoris in patients with HCM causing pain at rest or during variable levels of effort.

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Project Description:

Angina pectoris is a frequent symptom in patients with hypertrophic cardiomyopathy (HCM), often with atypical features such as variable threshold of onset and prolonged duration. To study whether vasoconstrictor stimuli affect coronary flow dynamics, anterior circulation coronary flow (cF, ml/min) was measured by thermodilution at rest and during pacing, before and after ergonovine (E) 0.15 mg intravenously in 23 patients with HCM and normal epicardial coronary arteries.

	HR	BP	cF	AVO_2	AP
Rest	78±12	92±14	92±30	11.1±1.7	0
Pacing	133±17	103±21	154±47	11.4±2.0	18/23
Rest	80±12	94±14	92±33	11.6±1.9	0
E	81±13	101±15+	96±35		0
Pacing	138±12	111±17+	134±47*	12.1±2.4	21/23

Values=mean ± standard deviation, *=$p<0.001$ vs control pacing, +=$p<0.001$ vs rest blood pressure (BP). HR=heart rate. Paced HR represents highest coronary flow before and after ergonovine. AVO_2=arterial - great cardiac vein O_2 content. AP=anginal pectoris.

Ischemia severity (as assessed by lactate metabolism) was unaltered by ergonovine and no epicardial coronary artery narrowing occurred during ergonovine. Thus peak coronary flow decreases with ergonovine, probably due to vasoconstriction of a maximally dilated microvascular bed, or of prearteriolar small coronary arteries. These results suggest that small vessel coronary vasoconstriction may explain many of the atypical features of angina pectoris in patients with HCM.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04069-02-CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Intraoperative 2-D echocardiography in HCM during septal myotomy-myectomy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Barry J. Maron, M.D.	Head, Echocardiography Lab.	CB, NHLBI
Charles McIntosh, M.D.	Senior Surgeon	SU, NHLBI
Javier Arce, M.D.	Medical Technician	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab.	CB, NHLBI
Ivonne Wesley	Medical Technician	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
Heart Surgery Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Echocardiography

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.6	0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ventricular septal myotomy-myectomy operation for obstructive hypertrophic cardiomyopathy requires intimate knowledge of ventricular septal anatomy for successful outcome. Often routine echocardiography cannot provide reliable information regarding septal morphology because of technical limitations. Therefore, we utilized a sterilizable 5MHz two-dimensional echo transducer for intraoperative studies in 9 patients with hypertrophic cardiomyopathy; echos were performed by placing the transducer directly on the anterior surface of right ventricle. In 6 of 9 patients, preoperative transthoracic M-mode and 2-dimensional echos did not provide definitive assessment of ventricular septal thickness. In each of these patients, measurements of ventricular septal thickness varied by 5-11 mm (average 7) due to suboptimal visualization of ventricular septal endocardial surfaces. However, in each patient intraoperative echocardiography provided a clear definition of endocardial borders and more accurate definition of ventricular septal thickness. In one patients transthoracic echo underestimated ventricular septal thickness by 5 mm; in the remaining 2 patients ventricular septal thickness by transthoracic and intraoperative echo were similar. Intraoperative echocardiography performed after resection of ventricular septal muscle also provided direct morphologic assessment of the depth, length and width of the ventricular septal myotomy-myectomy resection.

Therefore, our initial experience indicates that intraoperative two-dimensional echocardiography is a valuable aid to the surgeon performing the ventricular septal myotomy-myectomy operation.

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Project Description:

Ventricular septal myotomy-myectomy is recommended to patients with obstructive hypertrophic cardiomyopathy who do not benefit from medical therapy for the purpose of relieving subaortic obstruction. Ventricular septal myotomy-myectomy is performed through an aortotomy which prohibits the surgeon from directly visualizing the entire operative site. Thus, precise knowledge of ventricular septal anatomy is crucial to successful operation. In order to better define septal morphology, we utilized a sterilizable Diasonics 5MHz two-dimensional echo transducer for intraoperative studies in 9 patients with hypertrophic cardiomyopathy; echos were performed by placing the transducer directly on the anterior surface of right ventricle. In 6 of 9 patients, preoperative transthoracic M-mode and 2-dimensional echos did not provide definitive assessment of ventricular septal thickness. In each of these patients, measurements of ventricular septal thickness varied by 5-11 mm (average 7) due to suboptimal visualization of ventricular septal endocardial surfaces. However, in each patient intraoperative echocardiography provided a clear definition of ventricular septal thickness. In one patient transthoracic echo underestimated ventricular septal thickness by 5mm; in the remaining 2 patients ventricular septal thickness by transthoracic and intraoperative echo were similar. Intraoperative echo performed after resection of ventricular septal muscle also provided direct morphologic assessment of the depth, length and width of the ventricular septal myotomy-myectomy resection. In 8 patients intraoperative echocardiography showed ventricular septal myotomy-myectomy to be adequate and mitral systolic anterior motion was abolished or greatly reduced; in the other patient ventricular septal myotomy-myectomy was narrow and shallow and systolic anterior motion persisted. Hence, intra-operative 2-dimensional echocardiography is a rapid and easily performed procedure which constitutes a valuable aid and guide to the surgeon performing ventricular septal myotomy-myectomy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04070-02-CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Left ventricular filling by pulsed Doppler echocardiography in HCM

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Barry J. Maron, M.D.	Head, Echocardiography Lab.	CB, NHLBI
Javier Arce, M.D.	Medical Technician	CB, NHLBI
Robert O. Bonow, M.D.	Head, Nuclear Cardiology	CB, NHLBI
Yvonne Wesley	Medical Technician	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Echocardiography

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.4	0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

There are few truly non-invasive tests available to measure left ventricular diastolic function in patients with cardiac disease. In this study we utilized pulsed Doppler to assess transmitral flow-velocity patterns and characterize left ventricular filling and relaxation in 67 patients with hypertrophic cardiomyopathy and 35 normal controls. Patients with hypertrophic cardiomyopathy differed distinctly from normals with prolonged rapid diastolic filling, shortened diastasis, enhanced filling during atrial systole, (reduced ratio of peak flow-velocity in early diastole to that in atrial systole) and prolonged isovolumic relaxation. Abnormal left ventricular filling was present in 48 (72%) of 67 patients with hypertrophic cardiomyopathy, but was more common in symptomatic (25/27, 93%) than asymptomatic patients (23/40; 57%; $p<0.01$) and more frequent in obstructive (17/19, 90%) than in nonobstructive hypertrophic cardiomyopathy (31/48, 65%); $p<0.05$).

These findings demonstrate that pulsed Doppler echocardiography may be used to quantitatively assess left ventricular function and that impairment in left ventricular filling and relaxation are common and clinically important abnormalities in a population of patients with hypertrophic cardiomyopathy.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Analysis of trans-mitral valve flow-velocity may provide useful data on left ventricular diastolic function. We utilized pulsed Doppler echocardiography to record trans-mitral valve flow-velocity in 67 untreated patients with hypertrophic cardiomyopathy and 35 normal controls of similar age and heart rate. Patients with hypertrophic cardiomyopathy differed distinctly from normals with prolonged rapid diastolic filling, shortened diastasis, enhanced filling during atrial systole (reduced ratio of peak flow-velocity in early diastole to that in atrial systole) and prolonged isovolumic relaxation.

Abnormal left ventricular filling was present in 48 (72%) of 67 patients with hypertrophic cardiomyopathy, but was more common in symptomatic (25/27, 93%) than asymptomatic patients (23/40; 57%; $<p0.01$) and more frequent in obstructive (17/19, 90%) than in nonobstructive hypertrophic cardiomyopathy (31/48, 65%; $p<0.05$). Hence, 1) pulsed Doppler echocardiography is a new and valuable noninvasive method for quantitatively assessing left ventricular diastolic function, 2) left ventricular diastolic abnormalities are commmon (72%) in hypertrophic cardiomyopathy, but most frequent in patients with symptoms or subaortic obstruction. These findings underline the frequency and importance of impaired left ventricular filling in a population of patients with hypertrophic cardiomyopathy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04071-02-CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Spontaneous progression of left ventricular hypertrophy during adolescence in HCM

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Barry J. Maron, M.D.	Head, Echocardiography Lab.	CB, NHLBI
Javier Arce, M.D.	Medical Technician	CB, NHLBI
Yvonne Wesley	Medical Technician	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Echocardiography

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.25	0.25

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
- [ ] (b) Human tissues
- [ ] (c) Neither
- [ ] (a1) Minors
- [ ] (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Twenty-one relatives of patients with documented hypertrophic cardiomyopathy were studied prospectively over a period of 5 years with M-mode and wide-angle two-dimensional echocardiography to evaluate the potential for progression of left ventricular hypertrophy. Although each of these subjects had no or minimal hypertrophy prior to adolescence, 11 showed marked increase in the magnitude and distribution of hypertrophy including 3 who progressed from a normal heart. These findings also emphasize that echocardiographic screening of pre-adolescent family members cannot definitively exclude hypertrophic cardiomyopathy.

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Project Description:

Hypertrophic cardiomyopathy has been considered to be a congenital cardiac malformation with left ventricular dypertrophy often evident shortly after birth. To determine whether left ventricular mass is altered early in life, 21 asymptomatic and untreated relatives of patients with with hypertrophic cardiomyopathy were studied serially with 1 and 2-dimensional echo; initially at age 8-15 years (mean 11) and most recently at 13-19 years (mean 15). At initial examination, 13 patients had structurally normal hearts and 8 had mild left ventricular wall thickening. Over a 3-5 year follow-up, 11 patients showed marked increase in the magnitude and in the distribution of left ventricular hypertrophy, including 3 patients with progression from a normal left ventricle (at ages 9-12) to wall thickness of 14-21 mm (ages 13-17). Left ventricular wall thickness increased as much as 150% (average 75%), from 13.8 ± 4 mm to 24.4 ± 1 mm ($p<0.001$). This increase was in the basal anterior septum (5 patients) or areas of left ventricle not detectable by M-mode echo - i.e., anterior free wall (3 patients), posterior or distal ventricular septum (3 patients). In each patient progression of left ventricular hypertrophy occurred during a period of considerable body growth, at 12-19 years of age, but was not due to left ventricular pressure overload (mitral systolic anterior motion was absent).

In conclusion, we have shown for the first time, that the morphologic expression of nonobstructive hypertrophic cardiomyopathy can spontaneously appear or progress substantially in genetically predisposed relatives during adolescence when body growth is accelerated. These findings also emphasize that echocardiographic screening of pre-adolescent family members cannot definitively exclude hypertrophic cardiomyopathy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04072-02-CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Obstruction in hypertrophic cardiomyopathy: analysis by Doppler echocardiography

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Barry J. Maron, M.D.	Head, Echocardiography Lab.	CB, NHLBI
John S. Gottdiener, M.D.	Guest Investigator	CB, NHLBI
Javier Arce, M.D.	Medical Technician	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab.	CB, NHLBI
Ivonne Wesley	Medical Technician	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Echocardiography Lab

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A major controversy persists in cardiology as to whether true outflow obstruction may exist in patients with hypertrophic cardiomyopathy and sub-aortic gradients. In this study range-gated Doppler echocardiography was utilized to investigate this problem in 50 patients with hypertrophic cardiomyopathy and 20 normals.

In obstructive hypertrophic cardiomyopathy, left ventricular ejection was characterized by early and rapid emptying (76±14% of aortic flow-velocity in the initial one-third of systole). The proportion of forward flow occurring after mitral-septal contact (and therefore concommitant with the gradient and elevated intraventricular pressure) was considerable, averaging over 40%.

Mid-systolic impedance to left ventricular outflow was suggested by the rapid deceleration in aortic flow-velocity concommitant with mitral-septal contact and premature partial aortic valve closure. Furthermore, left ventricular ejection was prolonged (384±40 msec) and the ventricle continued to empty and shorten during the period when both the pressure gradient and markedly elevated intraventricular pressures were present.

In contrast, patients with nonobstructive hypertrophic cardiomyopathy showed no evidence of impedance to left ventricular ejection. Aortic flow-velocity waveforms were similar to normals, with flow persisting to aortic valve closure; significant systolic anterior motion and partial mid-systolic aortic valve closure were absnet, and the systolic ejection period was normal (303±27 msec).

Hence, in patients with hypertrophic cardiomyopathy mitral valve systolic anterior motion constitutes the mechanical obstruction to left ventricular emptying. Gradients produced by this mitral valve motion appear to be of pathophysiologic importance since the left ventricle continues to contract in the presence of markedly elevated intraventricular pressures.

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Project Description:

To determine whether true obstruction to left ventricular ejection exists in patients with hypertrophic cardiomyopathy and subaortic gradients, we analyzed with pulsed Doppler echocardiography the patterns of left ventricular emptying in 50 patients with hypertrophic cardiomyopathy (20 with and 30 without evidence of obstruction) and in 20 normals. In obstructive hypertrophic cardiomyopathy, left ventricular ejection was characterized by early and rapid emptying (76±14% of aortic flow-velocity in the initial one-third of systole). The proportion of forward flow-velocity occurring before initial mitral-septal contact (and, hence, before onset of the subaortic gradient), was variable, but averaged 58%. Conversely, the proportion of forward flow occurring after mitral-septal contact (and therefore concommitant with the gradient and elevated intraventricular pressure) was considerable, averaging over 40%.

Mid-systolic impendance to left ventricular outflow was suggested by the rapid deceleration in aortic flow-velocity concommitant with mitral-septal contact and premature partial aortic valve closure. Furthermore, left ventricular ejection was prolonged (384±40 msec) and the ventricle continued to empty and shorten during the period when both the pressure gradient and markedly elevated intraventricular pressures were present. In 16 of 20 patients a relatively small second peak in flow-velocity appeared in late systole. Since marked systolic anterior motion of the mitral valve was still present, the late systolic portion of forward flow-velocity also appeared to be largely ejected during imposition of a mechanical impediment to outflow.

In contrast, patients with nonobstructive hypertrophic cardiomyopathy showed no evidence of impedance to left ventricular ejection. Aortic flow-velocity waveforms were similar to normals, with flow persisting to aortic valve closure; significant systolic anterior motion and partial mid-systolic aortic valve closure were absent, and the systolic ejection period was normal (303±27 msec).

We conclude that in hypertrophic cardiomyopathy: 1) mitral valve systolic anterior motion produces a mechanical obstruction to left ventricular emptying, and a considerable portion of the stroke volume is impeded in its egress from the left ventricle; 2) gradients appear to be of pathophysiologic importance since the left ventricle continues to contract in the presence of markedly elevated intraventricular pressures; and 3) left ventricular ejection characteristics differ markedly between patients with and those without subaortic gradients.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04074-02-CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Chronic effects of amiodarone in patients with hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously found that high dose oral amiodarone (1200-1600 mg/day for 10 days) improves cardiac symptoms, prolonges exercise capacity, and reduces significant ventricular arrhythmias in patients with nonobstructive hypertrophic cardiomyopathy and severe cardiac symptoms. The present investigation was designed to assess the chronic effects of amiodarone in the same patient group while on lower dose maintenance therapy (400-600 mg/day for 2 months). Efficacy was determined by exercise testing, ambulatory ECG monitoring, radionuclide cineangiography, and semiquantitative symptoms score analysis. We found that exercise capacity improved significantly during chronic oral therapy in association with a reduction in the semiquantitative symptoms scores. The clinical responses were varied; one patient died suddenly despite improved symptoms and no ventricular arrhythmias, two others required the addition of calcium channel blockers, one has progressed to left ventricular myectomy, and 6 patients had satisfactory symptom control. It is our impression that amiodarone may prove to be a significant tool in the pharmacologic armamentarium in the treatment of patients with hypertrophic cardiomyopathy. In addition to its important effects on both supraventricular and ventricular arrhythmias, amiodarone improves cardiac symptoms and restores functional capacity in many patients with hypertrophic cardiomyopathy who have been refractory to standard medical including calcium channel blockers and beta blockers.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

To assess the chronic effects of oral amiodarone (A) in patients with hypertrophic cardiomyopathy (HCM) and refractory cardiac symptoms (Sx), maintenance dose A (A2=400-600 mg/day for 2 months) was compared with loading dose A (A1=1200-1600 mg/day for 10 days) and with no medications in 10 patients (7 nonobstructive and 3 obstructive). Efficacy was determined by exercise testing, ambulatory ECG-monitoring, radionuclide cinengiography and semi-quantitative Sx scores. Data = mean±SD.

	HR	ExD	R-EF	R-PFR	R-TPF	PVC's
No meds	78±18	209±174	70±14	2.8±1.0	204±50	263±585
A1	69±10*	442±207+	71±14	3.1±1.0	204±54	6±14*
A2	70±12	411±184**	74±9	3.4±1.1	189±41	2±2*

*p<.05, **p<.02, +p<.005 vs No meds. ExD=exercise duration (secs), R=rest, EF=ejection fraction (%), PFR=peak filling rate (end-diastolic volumes/sec), TPF=time to PFR (msec), PVC's=complexes/24 hrs.

Sx scores (0=no Sx thru 63=max Sx) decreased from 36±11 before A to 22±13 after A1 (p<.005) and to 26±13 after A2 (p<.05). Diastolic filling improved (either increased PFR and/or decreased TPF) in 8/9 patients after A2. Improved exercise capacity, and Sx scores (after A1 and A2) correlated poorly with LV functional changes, ΔHR and A blood levels. Clinical responses were varied; 6 patients had satisfactory control of Sx, 1 died suddenly despite improved Sx and no ventricular arrhythmias during A, 2 have required addition of calcium channel blockers, and 1 other progressed to operation.

We conclude that in patients with HCM, chronic A therapy usually prolongs exercise capacity, improves Sx, and abolishes ventricular arrhythmias. Thus, A may be useful in patients whose Sx are refractory to standard medical therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-04077-02-CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Amiodarone improves rapid diastolic filling in patients with HCM

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Robert O. Bonow, M.D.	Head, Nuclear Cardiology	CB, NHLBI
Cynthia M. Tracy, M.D.	Sr. Clinical Staff Fellow	CB, NHLBI
Michael V. Green, M.S.	Head, Imaging Physics Sec.	NM, CC
Stephen L. Bacharach, Ph.D.	Physicist	NM, CC
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
Nuclear Medicine Laboratory

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md.

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Although we have demonstrated that amiodarone improves exercise capacity, reduces cardiac symptoms, and diminishes the frequency and severity of complex ventricular arrhythmias in patients with hypertrophic cardiomyopathy, its mechanisms of action have been poorly defined. Therefore, we studied 30 patients with hypertrophic cardiomyopathy using radionuclide angiographic techniques before and after loading dose (1200-1600 mg/day for 10 days) and maintenance dose (400-600 mg/day for 2 months) amiodarone. We examined left ventricular functional changes both during systole and diastole. Left ventricular rapid diastolic filling was assessed by examining the peak rate of LV filling and the magnitude of LV stroke volume filled during rapid diastolic filling. We found that the vast majority of patients manifested an improvement in both the rate and magnitude of rapid diastolic filling after initial amiodarone therapy. Although changes during maintenance amiodarone therapy were less striking, all patients in whom the peak rate of filling or the magnitude of filling decreased compared to loading dose therapy also had reduced exercise capacity and worse cardiac symptoms. Thus, it appears that amiodarone improves diastolic filling characteristics in patients with hypertrophic cardiomyopathy and the changes in rapid diastolic filling parallel clinical status. This data suggest that improved rapid diastolic filling may be an important mechanism contributing to the clinical efficacy of amiodarone therapy in such patients.

Project Description:

To investigate the mechanisms whereby amiodarone (A) improves cardiac symptoms and increases exercise capacity (Ex) in patients with hypertrophic cardiomyopathy (HCM), we studied left ventricular (LV) function by radionuclide angiography in 30 patients before (C) and after loading dose A (A_1=1200-1600 mg/day for 10 days) and in 16 patients continued with maintenance dose A (A_2= 400-600 mg/day for 2 months). LV rapid diastolic filling (RDF) was assessed by peak rate of LV filling (PFR in end-diastolic volumes/sec) and as the percent of LV stroke volume filled during RDF (%RDF). Data=mean ±SD.

	HR	EF	PFR	% RDF
C	73±15	72±13	3.0±1.0	71±11
A_1	64±13+	72±13	3.5±11.4**	78±13*
A_2	69±16	71±12	3.3±1.1	76±13

p<.01*, .005**, .001 vs C. HR=heart rate, EF=% ejection fraction.

After A_1, PFR and %RDF increased in 75% and 70% of patients respectively. Changes in RDF during A_2 were less striking, but all patients in whom PFR and/or %RDF decreased compared to A_1 also had reduced Ex and worse cardiac symptoms. Thus, A improves the rate and magnitude of RDF in HCM and subsequent changes in RDF during A therapy relate to changes in clinical status. These data suggest that improved RDF is an important mechanism contributing to the clinical efficacy of A therapy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04079-02-CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The natural history of mitral regurgitation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Joann Urquhart, M.D.	Sr. Medical Staff Fellow	CB, NHLBI
Robert O. Bonow, M.D.	Senior Investigator	CB, NHLBI
Barry J. Maron, M.D.	Head, Echocardiography Lab	CB, NHLBI
Stephen Bacharach, Ph.D.	Physicist	NM, CC
Michael V. Green, M.S.	Head, Imaging Physics Sect.	NM, CC
Douglas R. Rosing, M.D.	Head, Cardiac Cath Lab	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
Nuclear Medicine Dept., Clinical Center, NIH

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.04	.04	

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☐ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Determining the optimal time for valve replacement surgery in patients with mitral regurgitation (MR) is an important clinical problem. Patients with few or no symptoms, despite severe mitral regurgitation, present a particular problem. Since some patients remain asymptomatic for 10-20 years, it would be unwise to subject a patient prematurely to an operation which has a high operative mortality (5-10%) or the risks of a prosthetic valve. On the other hand, waiting too long can increase the surgical mortality and jeopardize the postoperative effect, resulting in irreversible left ventricular dysfunction.

This protocol was initiated by the Cardiology Branch in 1978 to study the natural history of two groups of patients with MR; those who were asymptomatic with those who were operative candidates. Invasive (cardiac catheterization) and noninvasive (M-mode echo, stress tests and radionuclide angiography) parameters of LV function were performed in an effort to find an objective predictor of clinical deterioration.

What is clear so far is by the time patients with MR become symptomatic enough to require operation, most have sustained left ventricular dysfunction which appears to be irreversible. Preoperative left ventricular dilatation by M-mode echo (LVDD>70 or LVSD>45) may identify patients at greater risk for severe postoperative LV dysfunction. The clinical significance of this depressed postoperative LV function remains to be determined by long-term follow-up studies.

It is crucial that the nonoperated and the operated patients continue to have follow-up noninvasive studies to further determine what preoperative parameters influence post-operative clinical course and to prove the relationship of these findings to the natural history of MR patients postoperatively.

Project Description:

Previous studies have demonstrated that the LV ejection fraction (EF) of patients with mitral regurgitation deteriorates significantly following valve replacement. However, the long term clinical significance and effect on long-term LV function of this finding are unknown. We therefore studied the effects of mitral valve replacement on early postop (6 months to 2 years) and late postop (3 to 5 years, mean 3.5) LV function in 23 mitral regurgitation patients using radionuclide angiography. Before mitral valve replacement, ejection fraction was 48±8% (mean±SD); ejection fraction early after mitral valve replacement decreased to 35±13% ($p<0.001$) with 12 patients demonstrating >5% decrease in ejection fraction. The only patient who died during subsequent follow-up was the patient with the lowest ejection fraction early post-op (13%, compared to 35% preop). All remaining patients are alive; 19 of 22 have persistant improvement of >1 functional class compared to preop values. At 3-5 years ejection fraction was 39±14%, a significant increase ($p<.005$) from the early post-op studies; in 9 patients ejection fraction increased >5%. All patients with ejection fraction >30 on early study remained stable or showed an increase in ejection fraction on late study (43±9% to 50±10%, $p<.05$). However, of 8 patients with ejection fraction <30% on early study, 1 patient died, two had no change in ejection fraction between early and late studies, and 3 had a progressive decrease in ejection fraction of >5%. Hence, early post-op deterioration in ejection fraction does not generally correlate with subsequent clinical or LV functional course; most patients with substantial early decrease in ejection fraction manifest improved long-term LV function, with good clinical results 3-5 years postop. However, patients with profoundly decreased ejection fraction post-op (<30%) appear to constitute a group at risk of persistant LV dysfunction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL- 04089-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The use of a new low osmotic contrast agent, iopamidol, for angiography

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Carroll L. Hanson	Cath Lab Technician	CB, NHLBI
Richard O. Cannon, III, M.D.	Senior Investigator	CB, NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Cynthia M. Tracy, M.D.	Medical Staff Fellow	CB, NHLBI
Joann Urquhart	Staff Associate	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.1	.1	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Presently available contrast materials have a relatively high osmolality and are ionic in make-up. Recently, non-ionic contrast materials have been developed with lower osmolality, and thus offer the theoretical advantage of having fewer side effects as well as decreased or absent hemodynamic effects upon injection. Iopamidol is one such agent which is presently under investigational testing by E.R. Squibb and Sons, Inc.

We administered iopamidol to 20 patients for the purposes of left ventriculography and coronary angiography. Three of these patients had repeat left angiography, 4 also had aortic root angiography and one right ventriculography. Two patients experienced nausea with the initial LV injections, and in one patient, the same response occurred during injections of the left coronary artery. One patient also had a sneezing episode which was probably related to iopamidol administration and one developed a rash which may have been related to protamine administration. No patient had a significant hemodynamic or electrophysiologic side effect during or after iopamidol injection. Qualitative evaluation of angiographic results indicated adequate to superior quality of images.

In summary, iopamidol appears to be a safe contrast agent which provides good images. It appears that it might be a safer agent than those presently available.

170

Project Description:

Presently available contrast materials have a relatively high osmolality and are ionic in make-up. Recently, non-ionic contrast materials have been developed with lower osmolality, and thus offer the theoretical advantage of having fewer side effects as well as decreased or absent hemodynamic effects upon injection. Iopamidol is one such agent which is presently under investigational testing by E.R. Squibb and Sons, Inc.

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After iopamidol injection into either the LV or coronaries, 13 patients lengthened their RR interval by at least 10 msec and 11 decreased by at least 10 msec (9 patients showed both changes). The average maximal increase was 18 msec and decrease was 12 msec. Twelve patients decreased their T wave amplitude by at least 2mm while 10 increased it by at least 2mm (5 did both). The average maximal increase was 1.5mm and decrease was 1.8mm. Twelve patients increased their systolic blood pressure by at least 10mmHg, while 19/20 decreased their systolic blood pressure by at least 10mmHg during iopamidol administration. The average maximal increase and decrease was 16 and 19mmHg respectively. Only one patient experienced a decrease in LVEDP of >5mmHg with iopamidol, but 8 patients had an increase of $\geq$5mmHg with the average maximal increase being only 4.9mmHg.

In summary, iopamidol appears to be a safe contrast agent which provides good images. It appears that it might be a safer agent than those presently available.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04090-01 CB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Improved left ventricular function at rest after coronary artery bypass

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert O. Bonow, M.D.	Senior Investigator	CB, NHLBI
Vasken Dilsizian, M.D.	Dept. of Medicine-Georgetown Univ.	
Charles L. McIntosh, M.D.	Senior Surgeon	SU, NHLBI
Michael Jones, M.D.	Senior Surgeon	SU, NHLBI
Richard O. Cannon, M.D.	Senior Investigator	CB, NHLBI
Stephen L. Bacharach, Ph.D.	Physicist	NM, CC
Michael V. Green, MS	Head, Imaging Physics Sec.	NM, CC

COOPERATING UNITS *(if any)*

Surgery Branch, NHLBI
Department of Nuclear Medicine, CC

LAB/BRANCH

Cardiology Branch

SECTION

Nuclear Cardiology

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.4	.4	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Many patients with coronary artery disease manifest evidence for myocardial ischemia at rest, such as diastolic filling abnormalities and regional asynchrony, both of which may be reversed after successful percutaneous transluminal coronary angioplasty. Further evidence is the improvement in regional and global ventricular systolic function that occurs after coronary bypass operation. We observed a significant improvement in left ventricular ejection fraction at rest in 28 patients without previous myocardial infarction who were studied by radionuclide angiography before and 6 months after coronary bypass surgery. Moreover, quantitative regional analysis demonstrated improved regional function at rest after operation in those regions developing ischemia during exercise before operation. These data support the concept that many coronary artery disease patients have subclinical left ventricular ischemia under resting conditions which is reversible after restoration of coronary flow.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Successful coronary artery bypass surgery improves exercise induced left ventricular dysfunction in patients with coronary artery disease, but the frequency of improved left ventricular function at rest has not been established. To assess the influence of coronary artery bypass surgery on left ventricular function at rest, we studied 28 coronary artery disease patients without previous infarction by radionuclide angiography and coronary arteriography before and 6 months after coronary artery bypass surgery. None had angina at rest. Coronary artery bypass surgery significantly increased left ventricular ejection fraction during exercise (47±11 to 63±9%, $p<.001$), indicating reduction in reversible left ventricular ischemia. Moreover, left ventricular ejection fraction at rest increased (55±9 to 60±8%, $p<.005$), with 20 of 28 patients manifesting an increase compared to preoperative values: 12 of the 20 patients had apparently normal left ventricular function (ejection fraction and regional wall motion) before coronary artery bypass surgery. Quantitative regional left ventricular analysis indicated that improved ejection fraction at rest after coronary artery bypass surgery occurred in those regions developing ischemia during exercise before coronary artery bypass surgery. Thus, left ventricular global and regional function at rest improved, even in patients with apparently normal left ventricular function before coronary artery bypass surgery. These data support the concept that many coronary artery disease patients have subclinical left ventricular ischemia under resting conditions which is reversible after restoration of coronary flow.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL- 04091-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regional left ventricular asynchrony in coronary artery disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert O. Bonow, M.D.	Senior Investigator	CB, NHLBI
Dino F. Vitale, M.D.	Guest Worker	NM, CC
Stephen L. Bacharach, Ph.D.	Physicist	NM, CC
Michael V. Green, M.S.	Head, Imaging Physics Sec.	NM, CC
Steven M. Larson, M.D.	Chief, Nuclear Medicine	NM, CC

COOPERATING UNITS *(if any)*
Dept. of Nuclear Medicine, CC

LAB/BRANCH
Cardiology Branch

SECTION
Nuclear Cardiology

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.1	.1	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Global left ventricular relaxation and diastolic filling is abnormal in many patients with coronary artery disease who have normal left ventricular systolic function. In patients with normal regional wall motion, abnormal diastolic filling, assessed by radionuclide angiography, is related to diastolic asynchrony. In patients with visually apparent regional wall motion abnormalities, systolic asynchrony results in greater impairment in global diastolic filling, and the severity of asynchrony is directly related to the temporal delay in left ventricular filling. These data indicate that asynchronous left ventricular systolic function is a determinant of global abnormalities in left ventricular diastolic function.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

In coronary artery disease patients without systolic regional wall motion abnormalities, impaired global left ventricular left ventricular filling is related to regional diastolic asynchrony. To assess the additional importance of asynchronous left ventricular contraction on global diastolic filling at rest, we performed radionuclide angiography in 60 coronary artery disease patients with normal ejection fraction : 30 with and 30 without wall motion abnormalities. Ejection fraction, heart rate, and peak filling rate were not different among the 2 groups, but patients with wall motion abnormalities had greater prolongation of time to peak filling rate (185±27 vs 170±15ms, $p<.05$). Regional time-activity curves were derived by dividing the left ventricular region of interest into 20 sectors and also into 4 quadrants. Left ventricular systolic asynchrony was assessed by the regional variation in time to minimum volume about the global value (ΔTMV) and diastolic asynchrony by the regional variation in time to peak filling rate (ΔTPFR). Regional ΔTPFR was not different between the 2 groups (30±16 vs 26± ms, NS), but regional ΔTMV was greater in patients with wall motion abnormalities (43±21 vs 17±9 ms, $p<.001$) and correlated significantly with the prolongation of global time to peak filling rate ($r=.62$, $p<.001$). Hence, severity of diastolic asynchrony is similar in patients with and without wall motion abnormalities, but greater left ventricular systolic asynchrony in patients with wall motion abnormalities contributes importantly to the delay in global left ventricular filling.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04092-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Enhanced left ventricular function after distant coronary bypass

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert O. Bonow, M.D.	Senior Investigator	CB, NHLBI
Vasken Dilsizian, M.D.	Department of Medicine, Georgetown University	
Cynthia M. Tracy, M.D.	Sr. Clinical Staff Fellow	CB, NHLBI
Richard O. Cannon, M.D.	Senior Investigator	CB, NHLBI
Charles L. McIntosh, M.D.	Senior Surgeon	SU, NHLBI
Michael Jones, M.D.	Senior Surgeon	SU, NHLBI
Dino F. Vitale	Guest Worker	NM, CC
Stephen L. Bacharach	Physicist	NM, CC
Michael V. Green, M.S.	Head, Imaging Physics Sec.	NM, CC

COOPERATING UNITS *(if any)*
Department of Nuclear Medicine, CC
Surgery Branch, NHLBI
Dept. of Medicine, Georgetown University

LAB/BRANCH
Cardiology Branch

SECTION
Nuclear Cardiology

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.4	.4	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In patients with multivessel coronary artery disease, coronary bypass surgery often cannot be performed on all involved arteries. We therefore evaluated whether coronary artery bypass can, through collaterals, improve function in left ventricular myocardium served by nonbypassed arteries. Improvement in regional and global left ventricular function during exercise after operation was equivalent in patients in whom all bypass grafts were patent and those with only partially bypassed arteries. Exercise wall motion abnormalities improved and regional ejection fraction increased in 10/11 patients in regions that were not revascularized. Improved collateral perfusion was documented by angiography in 8 of these 10 patients. Hence, many patients with a nonbypassable coronary artery may still benefit from coronary artery bypass surgery, if the jeopardized myocardium is perfused by collateral vessels supplied by a stenosed artery amenable to bypass surgery.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

To determine whether coronary artery bypass can, through collaterals, improve function in left ventricular regions not revascularized (because of non-bypassable arteries or occluded grafts), we studied 21 patients with multivessel coronary artery disease by radionuclide angiography and coronary arteriography before and 6 months after coronary artery bypass. Left ventricular regional ejection fractions were computed by dividing the left ventricular region of interest into 20 sectors. For the total group, coronary artery bypass significantly increased global left ventricular ejection fraction during exercise (40 ± 13 to $51\pm14\%$, $p<.001$) and the change in ejection fraction from rest to exercise (-8 ± 10 to $2\pm6\%$, $p<.001$). Such improvement was observed in 8/10 patients with all stenoses bypassed, but also to an equivalent degree in 9/11 patients with only partially bypassed arteries. In 10 of these 11 patients, exercise wall motion abnormalities improved and regional ejection fraction increased (from 49 ± 15 to $65\pm16\%$, $p<.001$) in regions that were not revascularized. Improved collateral perfusion in these regions were demonstrated in 8/10 patients. Hence, many patients with a non-bypassable coronary artery may still benefit from coronary artery bypass, if the jeopardized myocardium is perfused by collateral vessels supplied by a stenosed artery amenable to bypass surgery.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL- 04093-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Left ventricular ejection dynamics in hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert O. Bonow, M.D.	Senior Investigator	CB, NHLBI
Cynthia Crawford-Green, M.D.	Medical Staff Fellow	CB, NHLBI
Sandro Betocchi	Guest Worker	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Barry J. Maron, M.D.	Senior Investigator	CB, NHLBI

COOPERATING UNITS *(if any)*
Nuclear Medicine Department, CC

LAB/BRANCH
Cardiology Branch

SECTION
Nuclear Cardiology

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.2	.2	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We investigated the dynamics and timing of left ventricular ejection in patients with hypertrophic cardiomyopathy (HCM), compared to patients with valvular aortic stenosis (AS), using radionuclide angiography. Patients with obstructive HCM had prolongation of left ventricular ejection time that was equivalent to patients with AS but significantly greater than patients with HCM without gradients. Despite prolonged ejection times, obstructive HCM patients had a period of brief, rapid ejection, with time to peak ejection rate values less than normal and equivalent to patients without gradients. Of note, 40% of patients with AS had supranormal ejection fractions. Hence, 1) supranormal EF does not exclude the possibility of significant outflow obstruction in HCM or AS, and 2) the outflow gradient in HCM represents true impedance to left ventricular emptying which occurs after an initial period of rapid ejection.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Previous studies suggest that the LV outflow tract gradient (OTG) in hypertrophic cardiomyopathy (HCM) does not represent obstruction to LV ejection since LV function is hypercontractile and since the rate and timing of ejection are similar in patients with and without OTG. However, comparison to valvular aortic stenosis (AS), with true outflow obstruction, has not been performed. We therefore studied LV ejection at rest by radionuclide angiography in 50 normal subjects, 40 AS patients (OTG $\geq$50mmHg), and 150 HCM patients of whom 57 had significant OTG (>50mmHg) and 93 did not ($\leq$20mmHg). Despite critical OTG, 16 AS patients (40%) manifested supranormal ejection fraction (EF). EF, peak ejection rate (PER) and time to PER (measured from the R wave) were similar in HCM patients with and without OTG, and time to PER was less than normal. However, total ejection time (ET) was prolonged in HCM patients with OTG to an equivalent or greater degree than in AS patients. (data=mean$\pm$SD):

	EF (%)	PER (EDV/sec)	Time to PER (msec)	ET (msec)	RR (msec)
Normal	56$\pm$6	2.9$\pm$.6	182$\pm$22	342$\pm$30	812$\pm$126
AS	64$\pm$13*	2.9$\pm$.9	217$\pm$20*	365$\pm$33*	823$\pm$132
HCM:OTG	75$\pm$11*+	3.4$\pm$.7**	154$\pm$23*+	389$\pm$39*+	820$\pm$150
HCM:no OTG	70$\pm$13*	3.8$\pm$.9*+	163$\pm$28*+	333$\pm$40+	820$\pm$156

*p<.001 vs normal, +p<.001 vs AS.

Hence, supranormal EF does not exclude significant LV outflow obstruction, and is commonly seen in patients with AS. Moreover, HCM patients with OTG have early rapid ejection, but total ET is prolonged to an equivalent or greater degree than in AS patients. These data provide further evidence that the OTG in HCM represents true impedance to LV emptying which occurs after an initial period of rapid ejection.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04094-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Coronary flow reserve are after dipyridamole

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard O. Cannon, III, M.D.	Senior Investigator	CB, NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Joann Urquhart, M.D.	Staff Associate	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.05	0.05

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We and others have demonstrated limitation in coronary flow reserve of the coronary micro-circulation to be a frequent mechanism of angina pectoris in patients with angiographically normal epicardial coronary arteries. We have found that this limited flow reserve can be demonstrated during rapid atrial pacing, especially after ergonovine administration, associated with angina pectoris and metabolic and hemodynamic evidence of myocardial ischemia. Because pacing does not allow assessment of total transmural coronary flow reserve, a potent coronary arterial vasodilator (dipyridamole) was utilized to investigate peak flow reserve in patients with anginal pain despite normal epicardial coronary arteries. Twenty patients were identified as having abnormal vasodilator reserve on the basis of precipitation of typical chest pain after pacing following the administration of ergonovine. Compared to 11 patients without chest pain, these 20 patients had significantly lower coronary blood flow and higher calculated coronary resistance during pacing after ergonovine. After administration of dipyridamole, the same 20 patients had significantly less of an increase in transmural coronary flow compared to the patients with normal flow reserve based on the pacing study after ergonovine. Additionally, 13 of the 20 patients experienced severe chest pain during dipyridamole despite an increase in flow. Only one control patient experienced chest pain. Thus patients with impaired coronary flow reserve in response to increasing myocardial oxygen demands also have limitation of flow reserve in response to dipyridamole, suggesting that maximal vasodilator reserve is reduced. This is compatible with the concept of a coronary myocardial redistribution of coronary flow away from the endocardium to the epicardium because of narrowing of small prearteriolar coronary arteries.

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Project Description:

We have recently found that many patients with anginal pain despite normal epicardial coronary arteries have a limited coronary flow response to pacing, especially after ergonovine, associated with angina pectoris and metabolic and hemodynamic evidence of myocardial ischemia. To assess the effect of a potent coronary arterial vasodilator, dipyridamole 0.5 to 0.7 mg/kg was infused intravenously in 31 patients following a pacing study with ergonovine. Patients who developed angina pectoris during pacing after ergonovine were considered to have abnormal vasodilator reserve (AVR). Those without chest pain were controls (C). Coronary flow (cF) in ml/min was measured in the great cardiac vein by thermodilution.

		HR	BP	cF	CR
Baseline	C	84±9	97±10	66±13	1.53±.33
	AVR	91±9	103±11	67±16	1.61±.36
Pacing	C	150±0	110±14	130±55	.83±.32
After Ergonovine	AVR	150±6	116±12	90±20*	1.33±.24*
Baseline	C	83±13	108±14	73±15	1.53±.26
	AVR	88±10	111±12	67±14	1.73±.34
After dipyridamole	C	108±17	94±12	203±40	.48±.12
	AVR	107±15	100±13	141±29*	.76±.20*

HC=heart rate, BP=mean blood pressure, CR=coronary resistance (BP/cF), *=P<0.005 vs. control.

Additionally, 13 of the 21 AVR patient experienced severe angina pectoris during dipyridamole infusion despite increased flow. Only one control patient had chest pain. Those patients with impaired flow reserve in response to increasing myocardial oxygen demands after a vasoconstrictor agent, ergonovine, also had a limitation to flow reserve in response to dipyridamole, suggesting that maximal transmural vasodilator reserve is reduced. These results were also compatible with the concept of a transmural redistribution of blood flow away from the endocardium due to flow limitation by narrowing of small but prearteriolar coronary arteries.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04095-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Investigation of myocardial ischemia in hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard O. Cannon, III, M.D.	Senior Investigator	CB, NHLBI
Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Angina pectoris is a common symptom in both patients with and in those without obstruction to left ventricular outflow. The present study was designed to investigate coronary flow and myocardial hemodynamics and metabolism in such patients. Thirty-three HCM patients, 18 without obstruction and 15 with a greater than 30mmHg gradient in the left ventricular outflow tract, underwent study.
We found that patients with obstructive hypertrophic cardiomyopathy had higher coronary flow and myocardial oxygen consumption at rest and during pacing compared to patients without obstruction, probably due to higher left ventricular systolic pressure and myocardial oxygen requirements. Flow was lower at the onset of ischemia in patients without obstruction, suggesting more limited peak flow capacity; this possibly relates to left ventricular microvascular obstruction. The different coronary flow patterns between patients with and without obstruction further demonstrate that left ventricular outflow gradients produce important physiologic effects.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Angina pectoris is common to patients with hypertrophic cardiomyopathy. To examine whether the presence or absence of obstruction to left ventricular outflow affects coronary flow and myoacardial metabolism, 33 patients (18 without obstruction [HC-N], 15 with obstruction [HC-O] with gradient of 84±33mmHg) underwent pacing. Anterior left ventricular flow was estimated by great cardiac vein flow (ml/min).

	HR	CF	LVSP	MVO2	AP
HC-N	basal	81±27	135±16	9.1±3.6	0
HC-O	basal	105±22*	205±30*	12.0±4.2*	0
HC-N	131±16	129±42	134±24	13.5±4.5	14/18
HC-O	129±15	159±37*	172±30*	18.7±8.0*	11/15

Values=mean ± standard deviation; *=p<0.005 vs respective HC-N; HR=heart rate; CF=coronary blood flow; LVSP=Left ventricular systolic pressure. MVO2=myocardial oxygen consumption (ml O_2min).

At heart rate of 150, 31 of 33 patients had angina pectoris; 6 of 18 patients without obstruction and 10 of 15 with obstruction produced lactate. Thus, patients with obstructive hypertrophic cardiomyopathy have at higher coronary flow and myocardial oxygen consumption at rest and during pacing than patients without obstruction, probably due to higher left ventricular systolic pressure and resulting myocardial oxygen demand. The lower coronary flow at onset of ischemia in patients without obstruction suggests more limited peak flow capacity, possibly related to left ventricular microvascular obstruction. The different coronary flow patterns between patients with or without obstruction further demonstrates that left ventricular outflow gradients produce important physiologic effects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL- 04096-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effect of Surgical relief of obstruction in hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard O. Cannon, III, M.D.	Senior Investigator	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Charles L. McIntosh, M.D.	Senior Surgeon	SU, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
NONE

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.05	.05	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Surgical relief of left ventricular obstruction by left ventricular myotomy/myectomy or mitral valve replacement is a therapeutic option in patients with hypertrophic cardiomyopathy who are severely symptomatic and refractory to medical management. To determine the effects of surgical relief of left ventricular outflow obstruction in patients with hypertrophic cardiomyopathy, 5 patients were studied at rest and during atrial pacing before and after operation (septal myectomy in 4 and mitral valve replacement in 1). Coronary flow to the anterior left ventricular septum and free wall, the site of maximum hypertrophy in these patients, was assessed by thermodilution. In all 5 patients there was successful relief of rest left ventricular outflow tract gradient from a preoperative gradient of 85±36mmHg to 4±6mmHg postoperatively. Surgical relief of left ventricular outflow tract obstruction reduced left ventricular systolic pressure, coronary flow, and myocardial oxygen consumption at rest and during pacing, lowered left ventricular end-diastolic pressure following pacing, and improved angina threshold and metabolic evidence of ischemia. These results demonstrate the importance of left ventricular outflow tract gradients in hypertrophic cardiomyopathy.

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Project Description:

To determine the effects of surgical relief (septal myectomy or mitral valve replacement) of left ventricular outflow tract obstruction in patients with hypertrophic cardiomyopathy, 5 patients were studied at rest and during atrial pacing, before and after operation. Coronary flow (ml/min) to the anterior left ventricular wall and septum was assessed by thermodilution.

Before operation		gradient=85±36 mmHg			
	RP	CF	LVSP	MVO_2	Lact +
Rest	85±8	104±25	205±30	12.8±4.5	0
Pacing	104±8	188±45	172±30	24.5±7.0	4/5
After operation		**gradient=4±8 mmHg***			
Rest	89±12	77±8*	126±16*	8.9±1.4*	0
Pacing	101±16	137±19	131±17*	15.8±2.1*	1/5

*=$p<0.05$ vs respective preoperative value. Values=mean ± standard deviation. BP=mean blood pressure (mmHg), CF=coronary flow, LVSP=Left ventricular systolic pressure, MVO_2=myocardial oxygen consumption (mlO_2/min), lact + = lactate production.

All patients developed angina at a heart rate of 110-130 preoperatively; 4 out of 5 patients improved their anginal threshold postoperatively with 2 patients experiencing no chest pain at all during pacing after operation. Left ventricular end-diastolic pressure rose to 29±4 mmHg preoperatively and to 24±4mmHg* postoperatively. Thus, surgical relief of left ventricular outflow tract obstruction reduces left ventricular systolic pressure, coronary flow and myocardial oxygen consumption at rest and during pacing, lowers left ventricular filling pressures post-pacing and improves anginal threshold and metabolic evidence of ischemia. These results demonstrate the importance of left ventricular outflow tract gradients in hypertrophic cardiomyopathy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL- 04097-01 CB

PERIOD COVERED
October 1, 1985 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Carbon dioxide laser-tissue interactions: improved efficacy at short pulse durations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
David J. Underhill, M.D.	Medical Staff Fellow	SU, NHLBI
Paul D. Smith, Ph.D.	Senior Investigator	BEIB
Robert F. Bonner, Ph.D.	Senior Investigator	BEIB
William C. Roberts, M.D.	Chief, Cardiac Pathology Branch	PB, NHLBI
Richard E. Clark, M.D.	Chief, Cardiac Surgery Branch	SU, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies were performed to help define the effects of carbon dioxide lasers on human coronary arteries in air. Specifically, changes in lasing parameters have important influences on the efficiency and thermal tissue effects using carbon dioxide lasers. Fresh human coronary arteries were studied employing a uniform methodology and we have concluded that short pulse (<10-20 msec) CO_2 lasing generally results in better tissue ablation with symmetric craters associated with minimal circumferential charring and a small zone of thermal injury. Thus, short pulse CO_2 may be an appropriate laser source for in vivo angioplasty investigations.

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GPO 914-918

Project Description

Laser (L) coronary angioplasty in humans will require a precise understanding of specific tissue effects for varying L pulse powers. Fresh human cadaver coronary arteries were longitudinally incised and exposed in air to a CO_2 L at pulse durations (PD) ranging from .3 to 300 msec with a constant energy density of 60 mJ/mm^2. Tissue effects were analyzed using a uniform methodology including gross morphology, light microscopy, ocular micrometry, surface thermography and fast-reactive thermocouples placed on the adventitia. At PD <10 msec, 0.14 cm^3 of tissue was ablated per J delivered; there was localized surface temperature (T) diffusion with a peak T <50°C and adventitial T changes were <5°C with a rapid return to baseline. For PD >10 msec, less tissue ablation was observed with increased surface T diffusion and peak T >300°C for the highest PD; peak adventitial T changes were 12°C with a prolonged T relaxation. At the lowest PD, rounded symmetricx craters were formed with minimal circumferential charring, and a zone of thermal injury (consisting of coagulation necrosis, vacuolization, and tissue compression) extended laterally 20u. As PD increased, a progressive increase in thermal injury was observed, with intense charring throughout the crater and a zone of injury >300u for the highest PD. Based upon these studies, we conclude: (1) at constant energy, short-pulse (<10msec) CO_2 L results in improved tissue ablation with minimal thermal injury, and (2) these quantitative analyses have accurately defined the requirements for a CO_2 L delivery system for future in vivo investigations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04098-01 CB

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Spectroscopic analysis of human aorta as an aid in laser selection for angioplasty

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
David J. Underhill, M.D.	Medical Staff Fellow	SU, NHLBI
Paul D. Smith, Ph.D.	Senior Investigator	BEIB
Robert F. Bonner, Ph.D.	Senior Investigator	BEIB
Richard E. Clark, M.D.	Chief, Cardiac Surgery Branch	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI
Howard Kruth, M.D.	Experimental Atherosclerosis Lab.	NHLBI

COOPERATING UNITS *(if any)*
NASA-Goddard Space Flight Institute

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These experiments were performed in an attempt to see if atheroma have different spectroscopic characteristics than normal human aorta intima. Transmission and reflection spectroscopy was performed using integrating spears in 10 specimens of human cadaver aorta between 270 and 2500 nm. We concluded that no intrinsic chromaphore was present that would consistently differentiate atheroma from normal tissue and that the intense absorption in the ultraviolet and infra-red regions provides the best propect for localized plaque ablation while minimizing thermal damage to surrounding vessel wall.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Laser (L) angioplasty would be safer and more efficacious if L energy were selectively absorbed by atheroma (A) relative to normal intima (N). Absorption (Ab) by 10 um sections of human aorta intima (n=10) with A of variable composition (fatty, fibrous, calcified) and N was measured from 270-2500 nm using a Beckman spectrophotometer equipped with transmission and reflection integrating spheres. In both A and N, prominent features were water Ab peaks (200 cm^{-1} at 1950nm) and a broad protein and nucleic acid Ab band in the ultraviolet (500-800 cm^{-1} at 275nm decreasing to 160-240 cm^{-1} at 310nm). Minor Ab peaks were observed in the visible region at 410, 540, and 575nm due to hemoglobin in unwashed samples and at 450nm in one fatty plaque sample. At each wavelength: the average of all A demonstrated no net preferential Ab compared with N and the Ab coefficient of any A was never greater than twice N. We conclude that (1) there is no intrinsic chromophore which will consistently differentiate A from N and (2) the intense Ab in the ultraviolet and in the infrared provide the best prospect for highly localized plaque ablation while minimizing thermal damage to surrounding vessel wall. Thus, ultraviolet (Excimer) and infrared (CO_2) L are best suited for angioplasty.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04099-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Disadvantages of Argon Lasers for Angioplasty

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
David J. Underhill, M.D.	Medical Staff Fellow	SU, NHLBI
Paul D. Smith, Ph.D.	Senior Investigator	BEIB
Robert F. Bonner, Ph.D.	Senior Investigator	BEIB
William C. Roberts, M.D.	Chief, Cardiac Pathology Branch	PB, NHLBI
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COOPERATING UNITS *(if any)*
National Cancer Institute

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This work was performed to help define the characteristics associated with argon ion lasers which are the most frequently used laser source in animal and human research for intravascular tissue ablation. Argon lasers were studied in air, blood, saline, and saline blood mixtures in an attempt to define the mechanism of tissue ablation and sorrounding thermal difusion characteristics. A uniform methodology was employed including careful ocular michrometry and thermographic analyses utilizing adventitial mechanical thermocouples and fast infra-red surface thermography. We found that argon lasers cause nonuniform large-step explosive vaporization of tissue followed by tissue combustion which is associated with marked radial thermal diffusion. Argon lasers appear to be a disadvantageous source for angioplasty given the lack of precision, marked variability in ablative effects, and widespread thermal diffusion to sorrounding normal tissues.

Project Description:

An evaluation of Argon laser (Ar) interactions with fresh human cadaver coronary arteries (n=8, target sites=96) provides evidence for serious limitations which may compromise the safety and efficacy of Ar angioplasty. Using continuous Ar through a 600 um fiber with varying parameters (.65-3w for 1-60s, fiber-target distance=1-4mm, media=air, saline, and blood) we found: (1) Explosive Tissue Ablation. Infrared surface thermography and histology indicate that initial crater formation is an inefficient, single large-step process resultingd from explosive vaporization of tissue water after surface temperature exceeds 100°C with liberation of gaseous and particulate by-products. (2) Marked Thermal Diffusion. Intense crater charring was surrounded by a broad transmural histologic zone of injury (zoi); from ocular micrometry, zoi surface area averaged >8x crater surface area. Histologic thermal injury occurred at intimal and adventitial temperatures above 45°C and required only 4% of the energy necessary to initiate tissue ablation. (3) Variable Thermal and Ablative Effects. Lasing efficiency and thermal diffusion varied with power density, media, fiber-target distance, and plaque composition. Hence, Ar angioplasty is nonselective for plaque, imprecise, associated with severe thermal injury, and results in variable ablative effects which may lead to vessel wall perforation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04100-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Animal models of atherosclerosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
David J. Underhill, M.D.	Medical Staff Fellow	SU, NHLBI
Joseph Pierce, DVM	Senior Staff Fellow	OD, NHLBI
Richard E. Clark, M.D.	Chief, Cardiac Surgery Branch	SU, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To complement ongoing studies including angioscopy and Laser-tissue interactions, it has become necessary to develop a reproducible animal model of atherosclerosis. To test prototype multifunctional catheters in an attempt to simulate uniclinical conditions, we have employed a variety of models in the animal laboratory. Thusfar, 14 miniature swine have been placed as combination of atherogenic diet (cholesterol levels 500-1,000 mg/dl mg/dl) and intravascular balloon endothelial denudation performed under fluoroscopy utilizing sterile surgical techniques. From a single femoral arterial cutdown, balloon denudation was performed in carotid and iliofemoral arteries bilaterally. Angiograms obtained immediately after baloon-induced barotrauma revealed marked vasospasm of the affected vessels. We have examined several animals at one-three months after the initial procedure who have died for various reasons during the maturation phase of the study. Intense and advanced focal atherosclerotic lesions were observed at the sites of balloon denudation consisting of calcified, organized thrombus and "human" appearing atherosclerotic morphology. In addition, we have performed balloon denudation in the aorta and iliofemoral system of 20 New Zealand white rabbits also receiving an atherogenic diet. Necropsy specimens in several rabbits who died weeks after the procedure, revealed less intense fatty streak lesions in the aorta with more focal hemodynamically significant abnormalities in the iliac vessels. We believe that these two models of atherosclerosis are well suited to help study the feasibility of in vivo laser angioplasty.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

To test the hypothesis that in vivo laser angioplasty can result in tissue ablation without disadvantageous thermal injury to sorrounding endothelium, animal models of atherosclerosis have been developed. Fourteen miniature swine were fed a highly atherogenic diet (casein, butter, peanut oil, cholesterol, and sodium cholate) and subjected to balloon intravascular barotrauma for the purpose of eliciting focal atherosclerotic reactions in response to endothelial denudation. From a single femoral arterial cutdown, utilizing sterile surgical technique and fluoroscopic control, balloon denudation was accomplished in carotid and iliofemoral vessels bilaterally in all animals. Angiography performed immediately after ballon denudation demonstrated marked large vessels spasm in the region of barotrauma. Thus far, three swine have died for a variety of reasons from 1 month to 3 months after this procedure Necropsy studies have revealed striking atherosclerotic lesions localized to the area of balloon catheter manipulation and consisting of calcified organized thrombotic lesions. These hemodynamically significant (human-like) atherosclerotic narrowings would appear well suited for our goals to test the use of laser techniques to remodel peripheral and coronary arteries. In addition, 20 New Zealand white rabbits were also fed an atherogenic diet and subjected to balloon denudation of the aorta (descending thoracic and abdominal) and iliofemoral vessels. Several of these rabbits have died subsequent to the procedure and histologic as well as gross morphologic examinations show less significant atherosclerotic lesions consisting mostly of foam cell and fatty streak abnormalities. We are hopeful that these two animal models will provide the substrate for a future study of angioscopy and laser techniques in cardiovascular therapeutics.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04101-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Comparison of XeCl and KrF Excimer Lasers

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
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Robert F. Bonner, Ph.D.	Senior Investigator	BEIB
William C. Roberts, M.D.	Chief, Cardiac Pathology Br.	PB, NHLBI
Richard E. Clark, M.D.	Chief, Cardiac Surgery Br.	SU, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
Naval Research Laboratories

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md.

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This work compares the effects of two forms of ultraviolet lasers (KrF and XeCl) on fresh human cadaver coronary arteries in an attempt to see if differences due to stronger protein and nucleic acid absorption in the mid UV range would favor the 248nm KrF laser for subsequent in vivo investigation. We found that both excimer lasers produced selective ablative tissue effects with minimal thermal difusion. However, the initial ablative threshold and near maximal ablative efficiency required more than twice the energy density with XeCl compared to KrF lasers. This may have important implications in the ultimate choice of a laser source. Improved ablative effects with KrF probably results from enhanced linear absorption but need to be viewed in the context of great mutagenicity and lower fiber damage threshold at 248nm which may offset such advantages.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

The pulsed excimer (Ex) laser (L) is a new L source for experimental coronary angioplasty. To determine if there are differences among ExL due to stronger protein and nucleic acid absorption at 248nm vs 308nm, human cadaver coronary arteries (n=8, target sites=114) were exposed to KrF (248nm) and XeCl (308nm) at varying energy densities (ED) to quantitatively assess thermal and ablative tissue effects (TE). Both ExL produced similar histologic TE; sharp, symmetric triangular craters without charring or surrounding thermal injury. However, at higher ED, XeCl moreso than KrF formed eccentric craters with lateral clefts which may result from shock wave effects. Infrared surface thermography and fast-reactive thermocouples on the adventitia demonstrate for both ExL minimal surface temperature changes (T) ($<20^{\circ}C$ at the crater base) with rapid thermal relaxation (<.5 sec) and almost no transmural T ($<2^{\circ}C$ on the adventitia). By ocular micrometry, initial ablative threshold and near maximal ablative efficiency of XeCl required ED > 2x that of KrF. These data suggest that (1) both XeCl and KrF produce highly localized TE with minimal thermal injury, and (2) KrF may have slight advantages in histologic crater appearance and ablative efficacy resulting from enhanced linear absorption. However, greater mutagenicity and decreased fiber damage threshold at 240nm may offset these advantages.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04102-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Optimal lasers for coronary angioplasty: tissue interaction studies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
David J. Underhill, M.D.	Medical Staff Fellow	SU, NHLBI
Paul D. Smith, Ph.D.	Senior Investigator	BEIB
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COOPERATING UNITS *(if any)*
NASA-Goddard Space Flight Institute
Naval Research Laboratories

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Laser-tissue interaction studies were performed to help define specific characteristics of laser sources which would be important for future animal and human investigations examining the feasibility of laser angioplasty. Fresh human cadaver coronary arteries were exposed to different lasers and differing lasing parameters and were studied employing a uniform methodology incorporating histology, ocular micrometry, fast-reactive mechanical thermocouples on the adventitia, and fast infrared surface thermography. This work has helped to delineate the tissue thermal injury patterns and ablative efficacy of various laser sources in air and will have major implications in deciding the proper specifications for a laser catheter system to be used in subsequent in vivo investigations.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Before laser (L) coronary angioplasty is considered in humans, efficacy and thermal effects associated with specific L sources and modes of delivery must be determined. Fresh human cadaver coronary arteries were longitudinally incised and exposed in air to different L. Tissue effects (TE) were analyzed using a uniform methodology incorporating gross morphology, light microscopy, surface thermography and fast-reactive thermocouples placed on the adventitia. L sources included pulsed CO_2, Nd-YAG (frequency doubled, mode-locked), Excimer (E)-XeCl and KrF, and continuous Argon (Ar). For each L studied, a wide range of energy densities and pulse settings were used resulting in TE which varied from superficial intima disruption to full thickness perforating craters. CO_2 caused variable TE; ↑pulse duration (>10msec) at constant energy resulted in ↓L efficacy, ↑charring, ↑zone of injury and ↑temperature changes (T). Ar was the least efficacious L studied; intense crater charring and a broad zone of injury were seen with the highest T. KrF was more efficacious tha XeCl, but both had localized TE and formed sharp, symmetrical triangular craters without charring or T and only a minimal zone of injury. Nd-YAG produced unusual TE; eccentric expanding craters with horizontal clefts in the media but without other significant injury, charring or T. We conclude: (1) these quantitative studies accurately define the requirements for L delivery systems in vivo, (2) these L sources have markedly different patterns of tissue injury and thermal diffusion, and (3) E and short-pulse CO_2 cause the most localized TE, and are the most efficacious; thus, these are preferable L sources for future animal and human investigations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04103-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
High resolution angioscopy: Feasibility, limitations and design considerations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
David J. Underhill, M.D.	Senior Staff Fellow	SU, NHLBI
Paul D. Smith, Ph.D.	Senior Investigator	BEIB
Robert F. Bonner, Ph.D.	Senior Investigator	BEIB
Richard E. Clark, M.D.	Chief, Cardiac Surgery Branch	SU, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

It is our impression that Laser angioplasty as a catheter-based technique for the treatment for coronary atherosclerosis will require the accurate identification of target lesions using ultrathin angioscopes. Therefore, to complement our work with laser tissue interactions we have studied commercial fibrotic endoscopes to study the vascular anatomy in 40 anesthetized animals and in 10 human cadaver preparations. Technical modifications were necessary to insure high resolution images and we found that arterial anatomy could be viewed in greater than 95% of vessels greater than 2mm in diameter. We also have attempted to define the design specifications required for the development of a multifunctional catheter system incorporating angioscopy and a fibrotic waveguide to provide the requisite energy densities of ultraviolet light which would result in effective and safe tissue ablation.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Commercial fiberoptic endoscopes (1.5, 1.8, and 2.5 mm OD) were used to study the vascular anatomy in 40 anesthetized animals (5 species) and 10 human cadaver preparations. Coupling lenses were designed to interface these endoscopes with a color video system. Balloon-tipped guiding catheters (2.0 to 4.0 mm OD) were fabricated to ensure occlusion of antegrade blood flow in peripheral arteries and to allow simultaneous infusion of saline solution which inhibited retrograde collateral flow. Coronary arteries and saphenous vein grafts were also visualized in patients during cardiopulmonary bypass in arrested hearts with an aortic cross clamp applied. Fresh human cadaver coronry arteries were incised and exposed in air to excimer lasers (XeCl 308 nm and KrF 248 nm) and tissue effects were studied using a uniform methodology including gross morphology, histology, infrared surface thermography and fast rective mechanical thermocouples placed on the adventitia. A wide range of energy densities and pulse settings wee used for each laser to help characterize threshold and near maximal thermocouples placed on the adventitia. A wide range of energy densities and pulse settings were used for each laser tohelp characterized threshold and near maximal ablative effects. Fused silica fibers, 400-600 micron OD, were independently evaluated for damage thresholds and transmission efficiency at 308 nm.

In animals and human cadavers, excellent resolution of arterial anatomy was achieved in >2mm ID. Atheroma, thrombi, venous valves, and collateral circulations were identified in several experiments. The major impediments to good visualization were excessive retrograde collateral blood flow and inability to steer the angioscope. Both XeCl and KrF excimer lasers caused highly localized tissue effects; sharp symmetric triangular craters were formed without surrounding histologic thermal tissue injury. Moreover, minimal adventitial ($<2^{\circ}C$) and surface ($<20^{\circ}C$) temperature changes were measured despite near transmural ablation in several cases. Concomitant bench tests revealed 60-70% transmission at 308 nm with a fiber damage threshold of 70 mJ/mm^2 (2-3 ns pulse duration). This exceeds the observed threshold in air for atheroma ablation of 32 mJ/mm^2 for XeCl (20 ns pulse duration and 5 mJ/mmm^2 for KrF (12 ns pulse duration.

High resolution angioscopy may be an important adjunct to identify potential targets and to direct laser ablation of atheroma. This requires a bloodless field, coaxial positioning of the endoscope, adequate illumination and high quality endoscopic and video equipment. Pulsed UV excimer lasers (XeCl 308 nm and KrF 248 nm) propagate without attenuation in the intra-arterial saline field required for in vivo angioscopy. Excimer laser energy was superficially absorbed and ablation of targets occurred with minimal damage to surrounding tissue. The direct visualization by angioscopy of atheroma during excimer laser ablation may lessen the risk of vessel wall perforation which remains a major clinical obstacle. These findings are important in the complicated task of designing a fiberoptic-laser catheter with the potential for intra-arterial ablation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL- 04104-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Abnormal fibrinolytic activity in patients with CAD: Improvement after Ancrod

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
University of Cincinnati Medical School, Hematology Division.

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this study was to define the frequency and reversibility of abnormal fibrinolytic activity in patients with coronary artery disease and non-acute ischemic chest pain syndromes. Measurements of tissue plasminogen activate (t-PA), inhibitor of tissue plasminogen activation (IPA) and alpha 2-antiplasmin were made in 10 patients with refractory angina before and after long-term treatment using Ancrod, which is a snake venom derivative possessing defibrinogenating and indirect tPA properties. We found that most patients with severe refractory angina have abnormal fibrinolytic activity either at rest or during exercise and that these abnormalities can be improved or normalized during Ancrod therapy. Moreover, Ancrod therapy also prolonged exercise capacity in most patients and reduced anginal symptoms. These preliminary observations suggest that primary or acquired deffects in fibrinolytic activity may play some role in ischemic chest pain syndromes and that such abnormalities can be improved with specific pharmaceutical agents having thrombolytic properties.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

To determine the frequency and reversibility of abnormal fibrinolytic activity in coronary artery disease, measurements of tissue plasminogen activator (tPA), inhibitor of tissue plasminogen activator (IPA) and alpha2-antiplasmin were made in 10 patients with refractory angina before and after long-term (for 2 weeks) thrombolytic therapy using Ancrod (A), a snake venom derivative with defibrinogenating and indirect tPA properties. Data obtained at rest were compared to a large normal population.

	tPA	IPA	alpha2-antiplasmin
Before A	4.4±5.4**	1072±359**	892±233*
After A	5.5±6.1**	808±330+	902±306*
Normals (n=219)	9.9±5.8	761±57	837±63

*p<.05, **p<.005 vs Normals, + p<.02 vs Before A, Data=mean±SD in activator or inhibitor units.

After A, all patients improved fibrinolytic activity (either increased tPA and/or decreased IPA). During A therapy, exercise capacity increased >50% in all patients and 3 patients had marked reduction in angina; however, changes in tPA or IPA after A correlated poorly with changes in exercise capacity, anginal symptoms, radionuclide angiography, great cardiac vein flow (at rest and during pacing) and metabolic indices of ischemia. Thus, in patients with coronary artery disease (1) fibrinolytic activity is frequently abnormal, manifesting as decreased tPA, increased IPA, and increased alpha2-antiplasmin, (2) thrombolytic therapy with A is associated with improvement in fibrinolytic activity (especially decreased IPA) and prolonged exercise capacity, (3) changes in fibrinolytic activity (before or after A) do not parallel standard indices of disease severity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL- 04105-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Abnormal fibrynolitic activity in patients with hypertrophic cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Douglas R. Rosing, M.D.	Head, Cardiovascular Diagnosis	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
University of Cincinnati Medical School, Hematology Division.

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Myocardial ischemia is an important associated event in patients with hypertrophic cardiomyopathy which may be accompanied by abnormal intramyocardial coronary arteries, coronary vasodilator reserve abnormalities, and abnormal myocardial perfusion scans (using Thallium-201). To examine the hypothesis that thrombogenic factors may contribute to ischemia in patients with hypertrophic cardiomyopathy, intrinsic fibrolytic activity was measured at rest and compared to a large normal population. We found that fibrinolytic activity was impaired in most patients with hypertrophic cardiomyopathy and that abnormalities in fibrinolytic activity were not associated with frequency or severity of multiple parameters of clinical disease. Long-term follow-up will be important to determine if these abnormalities in fibrinolytic activity are associated with a higher risk for intravascular thrombus formation which may result in ischemia, LV dysfunction, or sudden death.

Project Description:

Myocardial ischemia as well as abnormal intramyocardial coronary arteries (ICA) are common in patients with hypertrophic cardiomyopathy (HCM). We examined the hypothesis that thrombogenic factors may contribute to lumen narrowing of the ICA and ischemia by studying intrinsic fibrinolytic activity (FA) in 28 consecutive patients with HCM (average age=43 yrs). Measurements of tissue plasminogen activator (tPA), inhibitor of tissue plasminogen activation (IPA), and alpha 2-antiplasmin (AP) were obtained at rest and were compared to a large normal population (n=241).

	tPA	IPA	alpha 2-AP
HCM	4.4±4.3*	1027±198*	812±76
Normals	9.9±5.8	761±57	837±63

*p<.001 vs normals. Data=mean ±SD in activator or inhibitor units.

FA was impaired (decreased tPA and/or increased IPA) in 82% of HC patients. Abnormalities in FA were not associated with age, sex, ECG infarction, Echo LVH and LV function, cardiac symptoms including angina, or the presence of subvalvular obstruction. While changes in FA do not parallel other indices of disease activity, long-term clinical follow-up will be necessary to determine whether HCM patients with impaired FA are at greater risk for intravascular thrombus formation resulting in ischemia, LV dysfunction or sudden death.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04106-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Diastolic abnormalities in hypertrophic cardiomyopathy: relation to hypertrophy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Paolo Spirito, M.D.	Guest Investigator	CB, NHLBI
Barry J. Maron, M.D.	Head, Echocardiography Lab.	CB, NHLBI
Francesco Chiarella, M.D.	Department of Cardiology	Ospedale Galliera, Genoa, ITALY
Paolo Bellotti, M.D.	" " "	" "
Roberto Tramarin, M.D.	" " "	" "
Massimo Pozzoli, M.D.	" " "	" "
Carlo Vecchio, M.D.	" " "	" "

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Echocardiography Laboratory

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hypertrophic cardiomyopathy is characterized by both increased ventricular wall thickness and impairment to left ventricular filling. However, the interrelation between these two pathologic features of the disease is not well understood.

To investigate the relationship between diastolic abnormalities and left ventricular hypertrophy, 52 patients with hypertrophic cardiomyopathy and 22 normal subjects were studied with digitized M-mode echocardiography and two-dimensional echocardiography. Echocardiographic indexes of diastolic function were compared in patients with different extent of left ventricular hypertrophy.

Left ventricular relaxation and time to peak rate of increase in left ventricular dimension were prolonged (80±31 and 100±37 msec) in patients with hypertrophic cardiomyopathy and severe left ventricular hypertrophy, compared to patients with mild left ventricular hypertrophy (59±25 and 74±34 msec; $p<0.01$). However, left ventricular relaxation and time to peak rate of increase in left ventricular dimension were also abnormal in patients with mild left ventricular hypertrophy, compared to controls (24±12 and 49±12 msec; $p<0.01$). Furthermore, in 32 patients with hypertrophic cardiomyopathy who had normal poterior wall thickness, peak rate of posterior wall diastolic excursion was significantly reduced (11±3 cm/sec) compared to controls (16±2 cm/sec; $p<0.001$).

In conclusion, a mildly hypertrophied left ventricle and even non-hypertrophied left ventricular segments may demonstrate diastolic abnormalities in patients with hypertrophic cardiomyopathy. This finding suggests that the primary cardiomyopathic process in hypertrophic cardiomyopathy is not limited to areas of wall thickening.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:
To investigate the relationship between diastolic abnormalities and left ventricular hypertrophy, 52 patients with hypertrophic cardiomyopathy and 22 normal subjects were studied with digitized M-mode echocardiography and two-dimensional echocardiography. Echocardiographic indexes of diastolic function were compared in patients with different extent of left ventricular hypertrophy.

Time intervals from minimum left ventricular internal dimension to mitral valve opening and time to peak rate of increase in left ventricular internal dimension were significantly prolonged (80±31 and 100±37 msec, respectively) in patients with hypertrophic cardiomyopathy and the most extensive left ventricular hypertrophy, compared to patients with mild left ventricular hypertrophy (59±25 and 74±34 msec, respectively; $p<0.01$). Furthermore, peak rate of posterior wall diastolic excursion was significantly reduced in those patients with hypertrophic cardiomyopathy and posterior wall hypertrophy (8.3±4.0 cm/sec), compared to patients with hypertrophic cardiomyopathy but normal posterior wall thickness (11.2±3.4 cm/sec; $p<0.002$).

However, abnormal M-mode echocardiographic indexes of diastolic function were also identified in a substantial proportion of patients (i.e., 73%) with hypertrophic cardiomyopathy and only mild left ventricular hypertrophy. In these patients, time interval from minimum left ventricular internal dimension to mitral valve opening (59±25 msec) and time to peak rate of increase in left ventricular internal dimension (74±34 msec) were significantly different from normals (24±12 msec and 49±12 msec, respectively; $p<0.01$). Furthermore, in the 32 patients with hypertrophic cardiomyopathy who had normal posterior wall thickness, peak rate of posterior wall diastolic excursion was significantly reduced (11±3 cm/sec) compared to normals (16±2 cm/sec; $p<0.001$).

In conclusion, our findings show a relationship, in patients with hypertrophic cardiomyopathy, between magnitude of left ventricular hypertrophy and extent of diastolic wall motion abnormalities. However, our results also show that diastolic wall motion abnormalities are common in patients with hypertrophic cardiomyopathy and mild localized left ventricular hypertrophy and may even be identified in segments of the left ventricle which are of normal thickness. These data would suggest that the primary cardiomyopathic process in hypertrophic cardiomyopathy may not be limited to areas of gross wall thickening, and non-hypertrophied regions of the left ventricle may contribute to impairment of diastolic function in this disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04107-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Severe symptoms in patients with hypertrophic cardiomyopathy and mild hypertension

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Paolo Spirito, M.D.	Guest Investigator	CB, NHLBI
Barry J. Maron, M.D.	Head, Echocardiography Lab.	CB, NHLBI
Robert O. Bonow, M.D.	Head, Nuclear Cardiol. Lab.	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Echocardiography Laboratory

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In patients with the nonobstructive form of hypertrophic cardiomyopathy, a markedly hypertrophied noncompliant left ventricle is believed to be largely responsible for diastolic dysfunction and cardiac symptoms. However, we have recently shown that left ventricular diastolic impairment is also present in patients with hypertrophic cardiomyopathy who have only mild left ventricular hypertrophy. This finding would suggest that factors other than hypertrophy may play a role in the genesis of left ventricular dysfunction in patients with this disease.

To identify the pathogenetic mechanisms that, in the absence of marked left ventricular hypertrophy and subaortic gradient, may be responsible for left ventricular dysfunction in hypertrophic cardiomyopathy, we have analyzed the long-term clinical course of a population of patients with nonobstructive form of this disease and mild localized left ventricular hypertrophy. Fourty-four such patients were initially evaluated. Twelve of the 44 patients, who were identified as having severe cardiac symptoms (New York Heart Association functional class III or IV), comprise our study group. Radionuclide angiography showed depressed left ventricular systolic function in eight of the 12 patients (ejection fraction $\leq$45) and impaired diastolic filling in 10 (peak filling rate <2.5 EDV/sec). Long term M-mode echocardiographic follow-up (2-11 years; mean 6 years) available in 10 of the 12 patients, demonstrated progressive ventricular septal thinning (from 23$\pm$5mm to 19$\pm$4mm; $p<0.02$) and increase in left ventricular end-diastolic dimension (from 39$\pm$6mm to 46$\pm$5mm; $p<0.005$). Hence, severe cardiac symptoms may occur in patients with nonobstructive hypertrophic cardiomyopathy who have only mild localized left ventricular hypertrophy. Furthermore, the presence of progressive left ventricular wall thinning and cavity enlargement associated with depressed systolic function in these patients suggests that extensive myocardial scarring could be the determinant of their functional deterioration.

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Project Description:

In patients with the nonobstructive form of hypertrophic cardiomyopathy, a markedly hypertrophied noncompliant left ventricle is believed to be largely responsible for diastolic dysfunction and cardiac symptoms. However, we have recently shown that left ventricular diastolic impairment is also present in patients with hypertrophic cardiomyopathy who have only mild localized left ventricular hypertrophy. This finding would suggest that the primary cardiomyopathic process in hypertrophic cardiomyopathy may not be limited to areas of wall thickening, and that factors other than hypertrophy may play a role in the genesis of left ventricular dysfunction in patients with this disease.

In the present investigation, we have analyzed the long-term clinical course of a population of patients with nonobstructive hypertrophic cardiomyopathy and only mild localized left ventricular hypertrophy. Purpose of our study was to verify whether mild left ventricular hypertrophy may be associated with severe cardiac symptoms in hypertrophic cardiomyopathy and define which pathogenetic mechanisms, in the absence of marked left ventricular hypertrophy and subaortic gradient, may be responsible for left ventricular dysfunction.

Fourty-four patients with nonobstructive hypertrophic cardiomyopathy and mild localized left ventricular hypertrophy, as assessed with two-dimensional echocardiography, were initially evaluated. Twelve of the 44 patients, who were identified as having severe cardiac symptoms (New York Heart Association functional Class III or IV), comprise our final study group. Radionuclide angiography showed depressed left ventricular systolic function in eight of the 12 patients (ejection fraction $\leq$45) and impaired diastolic filling in 10 (peak filling rate <2.5 EDV/sec). Long-term M-mode echocardiographic follow-up (2-11 years; mean 6 years) available in 10 of the 12 patients, demonstrated progressive ventricular septal thinning (from 23$\pm$5mm to 19$\pm$4mm; $p<0.02$) and increase in left ventricular end-diastolic dimension from 39$\pm$6mm to 46$\pm$5mm; $p<0.005$). Hence, severe cardiac symptoms may occur in patients with nonobstructive hypertrophic cardiomyopathy who have only mild localized left ventricular hypertrophy. Furthermore, the presence of progressive left ventricular wall thinning and cavity enlargement associated with depressed systolic function in these patients, suggests that extensive myocardial scarring could be the determinant of their functional deterioration.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04108-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Assessment of diastole: Comparative analysis of Doppler and radionuclide angiography

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Paolo Spirito, M.D.	Guest Investigator	CB, NHLBI
Barry J. Maron, M.D.	Head, Echocardiography Laboratory	CB, NHLBI
Robert O. Bonow, M.D.	Head, Nuclear Cardiology Laboratory	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Echocardiography and Nuclear Cardiology Laboratories

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Doppler echocardiography is a new, noninvasive, and easily performed technique that provides important clinical information about valvular stenosis and regurgitation, intracardiac shunts and cardiac output.

This investigation was performed to determine whether variables obtained from the Doppler left ventricular diastolic flow-velocity profile provide an estimate of diastolic function. We compared measurements of diastolic flow-velocity obtained by Doppler echocardiography with volumetric measurements of left ventricular diastolic filling determined by radionuclide angiography in 12 subjects without cardiac disease and in 25 patients with a variety of cardiac diseases. Doppler echocardiography and radionuclide angiography showed agreement in distinguishing normal from abnormal diastolic function in 21 (84%) of the 25 patients with cardiac disease, diastolic function being identified as normal in 8 and abnormal in 13 of these patients. Furthermore, good correlations were observed between certain Doppler parameters of left ventriculardiastolic flow-velocity and radionuclide angiographic parameters of left ventricular filling. The time interval from the aortic closing component of the second heart sound to the end of the early diastolic flow-velocity peak, assessed with Doppler echocardiography, correlated well with the time interval from end-systole to the end of rapid filling, assessed with radionuclide angiography ($r=0.83$). Descent of the Doppler early diastolic flow-velocity peak correlated well with the radionuclide angiographic peak filling ($r=0.72$). Also, the ratio between the heights of the early and the late (due to atrial systole) peaks of diastolic flow-velocity showed good correlation with the ratio between percent of left ventricular filling during rapid filling and during atrial systole ($r=0.76$). These findings demonstrate that the left ventricular diastolic flow-velocity profile obtained with Doppler echocardiography compares favorably with radionuclide angiography in the evaluation of left ventricular diastolic function. Therefore, Doppler echocardiography may be useful as a noninvasive and easily performed method of estimating diastolic performance in patients with cardiac disease.

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Project Description:

This investigation was performed to determine whether variables obtained from the Doppler left ventricular diastolic flow-velocity profile provide an estimate of diastolic function. We compared measurements of diastolic flow-velocity obtained by Doppler echocardiography with volumetric measurements of left ventricular diastolic filling determined by radionuclide angiography in 12 subjects without cardiac disease and in 25 patients with a variety of cardiac diseases. Doppler echocardiography and radionuclide angiography showed agreement in distinguishing normal from abnormal diastolic function in 21 (84%) of the 25 patients with cardiac disease, diastolic function being identified as normal in eight and abnormal in 13 of these patients. Furthermore, good correlations were observed between certain Doppler parameters of left ventricular diastolic flow-velocity and radionuclide angiographic parameters of left ventricular filling. The time interval from the aortic closing component of the second heart sound to the end of the early diastolic flow-velocity peak, assessed with Doppler echocardiography, correlated well with the time interval from end-systole to the end of rapid filling, assessed with radionuclide angiography ($r=0.83$). Descent of the Doppler early diastolic flow-velocity peak correlated well with the radionuclide angiographic peak filling rate ($r=0.72$). Also the ratio between the heights of the early and the late (due to atrial systole peaks of diastolic flow-velocity showed good correlation with the ratio between percent of left ventricular filling during rapid filling and during atrial systole ($r=0.76$).

These findings demonstrate that the left ventricular diastolic flow-velocity profile obtained with Doppler echocardiography compares favorably with radionuclide angiography in the evaluation of left ventricular diastolic function. Therefore, Doppler echocardiography may be useful as a noninvasive and easily performed method of estimating diastolic performance in patients with cardiac disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL- 04109-01 CB

PERIOD COVERED
October 1, 1984 to September 31, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Myocardial Neovascularization with an Angiogenic Factor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Ellis F. Unger, M.D.	Medical Staff Fellow	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
Veterinary Resources Branch

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md.

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

One of the major problems in cardiology today is how to more effectively treat individuals with coronary artery disease who have symptoms refractory to conventional therapy, including antianginal drugs and coronary artery bypass surgery. One potential approach we are currently investigating is implantation of the internal mammary artery (IMA) into ischemic regions of the left ventricle. This operation has been performed on patients in the past, but the blood flow through the IMA was found to be generally insufficient, incapable of delivering enough blood flow to importantly influence symptoms.

A potent growth factor, known to cause the proliferation of blood vessels (angiogenesis factor) has recently been derived from the greater omentum of the cat. Our experiment is designed to assess the ability of this angiogenesis factor, as well as heparin (which has been shown to be angiogenic in vitro, to potentiate the growth of vascular connections derived from IMA's implanted in chronically ischemic myocardium in a canine model.

Foxhounds will undergo IMA implantation into the anterior wall of the left ventricle (Vineberg Procedure). Animals will randomly be assigned to receive continuous administration into the IMA of either angiogenesis factor, heparin, or normal saline. The area of the left ventricle in which the IMA graft is placed will be rendered ischemic over a two to four week period by positioning Ameroid constrictors around the left anterior descending coronary artery and the first marginal branch of the circumflex coronary artery. Animals will be studied eight weeks postoperatively to determine both the baseline myocardial blood flow and the maximum capacity for myocardial blood flow (vasodilator reserve) in the ischemic zone. The gross anatomic and microscopic distribution of vascular anastomoses as well as the density of coronary arteries within the ischemic area will subsequently be determined.

Project Description:

One of the major problems in cardiology today is how to more effectively treat individuals with coronary artery disease who have symptoms refractory to conventional therapy, including antianginal drugs and coronary artery bypass surgery. One potential approach we are currently investigating is implantation of the internal mammary artery (IMA) into ischemic regions of the left ventricle. This operation has been performed on patients in the past, but the blood flow through the IMA was found to be generally insufficient, incapable of delivering enough blood flow to importantly influence symptoms.

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Eighteen Foxhounds of either sex weighing 25-40 kg will undergo internal mammary artery implantation into the anterior wall of the left ventricle (Vineberg procedure) under general anesthesia. Animals will be randomly assigned to receive continuous intra-arterial administration of angiogenic factor, 1-2 ml/day, heparin sodium, 25-35 units/hr, or normal saline, 2-3 ml/day (control) into the tip of the IMA, accomplished utilizing a subcutaneous Infusaid pump. (Intermedics-Infusaid Corp., Norwood, Mass). Five animals will undergo IMA ligation after implantation and serve as shams.

The area of the left ventricle accepting the Vineberg graft will be rendered ischemic over a 2 to 4 week period by positioning Ameroid constrictors around the left anterior descending coronary artery after the takeoff of the first diagonal branch, and on the first marginal branch of the circumflex coronary artery. Animals will be studied at 8 weeks postoperatively. Regional myocardial flow will be determined with radiolabeled microspheres 14±1 um (3M Company) at baseline and during IMA occlusion. Maximal vasodilator reserve will be ascertained after dipyridamole 0.25 mg/kg IV, and flow to the anterior wall will be compared before and after IMA occlusion. IMA flow will also be measured wih an electromagnetic flow probe (Carolina Medical Electronics, Inc.) before and after dipyridamole administration.

After sacrifice the IMA will be perfused with radioopaque Microfil (Canton Biomedical Products, Inc., Boulder, CO) at systemic pressure. Radiographic and histologic studies will then be undertaken to ascertain the gross anatomic and microscopic appearance of the anastomosis.

Of 6 animals studied to date, 2 have been eliminated from further analysis due to complete graft failure and/or malposition of ameroids, undoubtedly secondary to operative technique. Preliminary results in the other 4 animals have been encouraging; however, it is too early to draw any conclusions from the limited data available. Eight additional animals have undergone surgery and are awaiting study.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL- 04110-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Electrical stimulation in patients with HCM at risk for sudden death

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Albert Del Negro, M.D.	Director, Electrophysiology Dept.,	Georgetown Univ.
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Martin B. Leon, M.D.	Senior Investigator	CB, NHLBI
Richard O. Cannon, M.D.	Senior Investigator	CB, NHLBI
Barry J. Maron, M.D.	Head, Echocardiography Lab	CB, NHLBI
Stephen E. Epstein, M.D.	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Of 15 patients enrolled in the electrophysiologic protocol for management of patients at high risk for lethal arrhythmias in hypertrophic cardiomyopathy, 4 (27%) have had ventricular tachycardia induced in the absence of medications. Two additional patients exhibited a pro-arrhythmic effect to a Type 1A anti-arrhythmic. Abnormal AV nodal function has been found in 4 patients, and abnormal sinus node function in 2 patients. Re-entrant arrhythmias have been documented in 2 patients, one of whom also had an atrial-ventricular bypass tract.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Programmed electrical stimulation with intracardiac electrogram recordings are being performed in patients with hypertrophic cardiomyopathy at high risk for sudden death. The purpose of these studies is to describe the electrophysiologic characteristics of these patients, to better define the possible contribution of ventricular arrhythmias to sudden death, and to develop more effective therapy.

To date 15 patients with HCM have been enrolled in the electrophysiologic protocol. All patients studied have had documented spontaneous ventricular arrhythmias ranging from nonsustained ventricular tachycardia, to ventricular fibrillation.

All patients underwent baseline electrophysiologic studies off all medications. Four of the 15 patients (27%) developed ventricular tachycardia on no medications. Only one of these patients degenerated to ventricular fibrillation.

Repeat electrical stimulation was performed in the laboratory following administration of intravenous medications. Three of the 4 patients who had initially developed ventricular tachycardia were non inducible on medications. The fourth patient could not receive a Type IA antiarrhythmic in the laboratory because of known drug intolerance. That patient is currently receiving oral mexiletine and is to undergo repeat electrophysiologic study once loaded orally. Two additional patients have had a pro-arrhythmic effect of pronestyl in the laboratory, emphasizing the need for acute drug testing.

At this point, 7 patients have been randomized to electrophysiologic guided therapy and 7 have been randomized to amiodarone treatment. Additional electrophysiologic data obtained has included abnormal AV nodal function in 4 patients, abnormal sinus node function in 2, and re-entrant arrhythmias in 2. The second patient with a re-entrant arrhythmia was also found to have an atrial ventricular bypass tract.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-04111-01 CB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Coronary flow reserve in dilated cardiomyopathy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard O. Cannon, III, M.D.	Senior Investigator	CB, NHLBI
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COOPERATING UNITS *(if any)*
None

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NHLBI NIH, Bethesda, Md 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.1	.1	

CHECK APPROPRIATE BOX(ES)
- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In the majority of patients with dilated cardiomyopathy the etiology is unknown. Many patients with dilated cardiomyopathy complain of anginal-type pain despite angiographically normal coronary arteries. To examine whether abnormalities in coronary flow exist in dilated cardiomyopathy, 20 patients with dilated cardiomyopathy and normal epicardial coronary arteries, 8 of whom had frequent chest pain, underwent measurement of great cardiac vein flow and myocardial metabolism at rest and during pacing to a heart rate of 150. After administration of ergonovine, all 8 patients with the history of chest pain experienced their typical chest pain. Compared to patients without chest pain, their coronary flow was lower and coronary resistance higher with significant widening of the AVO_2 difference suggestive of myocardial ischemia. Additionally, the increase in left ventricular filling pressures was higher in this group. There was no significant change in EKG or epicardial coronary luminal diameter by angiography. Administration of dipyridamole 0.5 to 0.75 mg intravenously to a subgroup of these patients suggested impairment in transmural coronary flow reserve compared to patients without chest pain after ergonovine administration. Thus, patients with dilated cardiomyopathy and chest pain by history may have limited coronary vasodilatory reserve, especially after vasoconstrictor stimulus. Whether this contributes to myocardial damage and dilated cardiomyopathy or is an epiphenomenon of an unrelated etiology, remains to be determined.

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Project Description:

In the majority of patients with dilated cardiomyopathy the etiology is unknown. Many patients with dilated cardiomyopathy complain of anginal-type pain despite angiographically normal coronary arteries. To examinate whether abnormalities in coronary flow exist in dilated cardiomyopathy, 20 patients with dilated cardiomyopathy (avg. ejection fraction 21%) and angiographically normal coronary arteries, 8 of whom had frequent chest pain by history, underwent measurement of great cardiac vein flow and myocardial oxygen and lactate consumption at rest and during pacing to a heart rate of 150. Pulmonary artery wedge pressure was measured at rest and post-pacing. During pacing alone, 5 patients with the history of angina pectoris had chest pain, associated with lower great cardiac vein flow than the 14 patients without pain (122±32 vs 153±32, non-significant). Repeat pacing study was performed after ergonovine 0.15 mg intravenously.

Rest	HR	BP	CF	CR
No AP	96±11	85±13	97±22	.95±32
AP	87±14	96±10	89±33	1.23±47
Pacing				
No AP	150±1	95±16	163±38	.61±.14
AP	150±0	104±9	115±30*	.97±.26**

*=$p<0.25$, **=$p<0.005$ vs no angina pectoris(AP) measurements. HR=heart rate, BP=mean blood pressure, CF=great cardiac vein flow, CR=coronary resistance (CB/CF).

All 8 patients with the chest pain by history experienced their typical chest pain during pacing after ergonovine despite no significant change in EKG or coronary artery luminal diameter by angiography. No chest pain was experienced by the patients without history of chest pain. Myocardial ischemia in the angina pectoris patients was suggested by fall on lactate consumption and widening of the AVO_2 difference and a greater increase in left ventricular filling pressures after pacing.

Dipyridamole 0.5 to 0.7.5 mg was administered intravenously to 16 patients with dilated cardiomyopathy. The 13 patients with no history of angina pectoris had a greater coronary flow reserve (224±71 ml/min) than the 3 patients with a history of angina (154±23 ml/min). Because of the small number of patients given dipyridamole, the difference was not statistically significant.

Thus, patients with dilated cardiomyopathy and chest pain by history may have limited coronary flow reserve, especially after vasoconstrictor stimulus. Whether this contributes to myocardial damage and dilated cardiomyopathy or is an epiphenomenon related to another etiology remains to be determined.

Annual Report of the Laboratory of Cell Biology,
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1985

Actin Polymerization: Dr. Korn has continued his studies of the role of ATP hydrolysis in the polymerization of actin, the major protein of the membrane-cytoskeletal complex of eukaryotic cells. Most of the work this year has been concerned with the elongation phase.

A new experimental method was developed this year, following polymerization under continuous sonication, which results in an increase in the concentration of filaments of constant length rather than an increase in the length of filaments of constant concentration, as occurs in the absence of sonication. In this way, the concentration of filament ends is greatly increased so that their properties can be studied.

The validity of the method was demonstrated by showing that the experimental data for the equilibrium polymerization of ADP-actin under continuous sonication exactly fit the theoretical curve, i.e. the polymerization curve was symmetrical about the mid-point, identical (when normalized) for all concentrations of ADP-actin, and linear on a semi-log plot. Moreover, a plot of the maximum rate of polymerization (i.e. the rate at $t_{1/2}$) extrapolated to zero at 8.3 μM actin, a value for the c_c of ADP-actin identical to that obtained by more traditional methods.

Because of the continual hydrolysis of ATP, the polymerization of ATP-actin is a steady state, not an equilbrium, process. Nonetheless, when ATP-actin at high concentration was polymerized under continuous sonication symmetrical polymerization curves were found to a transient plateau followed by a slow depolymerization, i.e. ATP-actin polymerization under these conditions was initially a pseudo-equilibrium process. This was because the rate of polymerization was so greatly accelerated by sonication that it was completed before significant ATP hydrolysis had occurred. Thus, an actin filament was transiently formed which contained essentially only ATP-subunits. Analysis of the data for this equilibrium polymerization of ATP-actin gave a c_c = 3 μM, much higher than the steady state of 0.35 μM for ATP-actin.

The fact that the c_c for the ATP-actin equilibrium polymer was much higher than for the ATP-actin polymer at steady state suggested that the ATP cap previously demonstrated to be present at steady state must be very short and that filaments with a short ATP-cap are more stable than filaments with a long stretch of ATP-subunits. This implies that the heterologous interaction between the ATP-actin and ADP-actin subunits at the interface between the ATP-cap and the ADP-body of the filament must be stronger than the homologous interactions between ATP-actin subunits only or between ADP-actin subunits only. It was found, in confirmation of the hypothesis, that the rate of nucleation and the rate of elongation were faster for copolymerization ATP-actin and ADP-actin than for polymerization of ATP-actin or ADP-actin alone.

These data, and data reported in previous years, led to a new hypothesis for the detailed events of actin polymerization in ATP. The end of the growing filament can be considered to have several possible configurations, considering only the three terminal subunits because all other subunits are in an environment

approximating that of the third subunit from the filament end. The following four confirmations are considered most likely:....DDD, ...DDT, ...DTT, and ....TTT. The relative concentrations of the four species will depend on the concentration of monomeric ATP-actin. When only monomeric ADP-actin is present only ....DDD ends are possible and these have a c_c of 8 μM. At very high concentrations of ATP-actin only ....TTT ends (and longer stretches of ATP-subunits) are possible and these have a c_c of 3 μM. At lower concentrations of ATP-actin, ...DDT and ....DTT become the dominant species and the c_c is 0.35 μM. Detailed mathematical treatment of this model produced computer-generated elongation curves that precisely fit the experimental data.

In the above discussion, the kinetics of actin polymerization was treated as if there were only one elongating end on the filament when, in fact, there are two. For the equilibrium polymer of ADP-actin (or ATP-actin), c_c will be the same at the two ends but for the steady state polymer c_c can be (and is) different at the two ends. For both equilibrium and steady state polymers, k_+ and k_- can be (and are) different at the two ends.

When polymerization kinetics are studied in bulk solution, the measured association and dissociation rate constants will be the sum of the values for the two ends and the measured critical concentration will be the sum of the dissociation rate constants divided by the sum of the association rate constants. To determine the individual values for each end it is necessary to block one end specifically thus allowing the values for the other end to be determined and, by difference, the value for the other end to be calculated. Dr. Korn used plasma gelsolin and the drug cytochalasin D to block the barbed ends of actin filaments and thus obtain kinetic values for the pointed ends alone to compare to the values for barbed plus pointed ends obtained in the absence of the barbed-end blockers. Furthermore, from the shape of the curves of elongation rate vs actin concentration when the barbed filament ends were blocked, it was deduced that the pointed end is not capped at the critical concentration in the presence of ATP although the barbed end is capped. At high actin concentrations, i.e. above the critical concentration the pointed end as well as the barbed end probably has an ATP-cap.

Regulation of Non-Muscle Myosins: Previous work has shown that myosin II consists of two heavy chains of 185,000 daltons and two pairs of light chains of 17,500 and 17,000 daltons. The heavy chains are phosphorylated at three serine residues that lie within an 11-amino acid sequence that begins just 8 residues away from the carboxyl tail and is at least 90 nm away from the ATPase and actin-binding sites that are regulated. Only the dephosphorylated myosin II has actin-activated ATPase activity. Because only filamentous forms of myosin II are enzymatically active, because phosphorylated myosin II can inactivate unphosphorylated myosin II in copolymers and because myosin II from which a carboxyl-terminal peptide of 66 amino acids (which included the phosphorylation sites) has been cleaved cannot form bipolar filaments and has little actin-activated ATPase activity, we previously proposed that phosphorylation regulates myosin II by affecting filament conformation.

Dr. Korn's group has now found conditions where trypsin produces a 68,000-dalton head-fragment free of the rest of the heavy chain. Complete Ca^{2+}-ATPase activity is retained but almost no actin-activated Mg^{2+}-ATPase activity remains in the 68-kDa head. The 68-kDa head has been crosslinked to F-actin by the zero-length crosslinker EDC, in about 20% yield. The crosslinked F-actin-head complex was found to have very high actin-activated ATPase activity. Thus, these data in-

dicate that the 68-kDa head is fully competent catalytically but that it may have much poorer affinity for actin than does the native molecule.

In other studies, the ATPase site was photoaffinity labeled by UV irradiation of myosin II in the presence of [^{3}H]-UTP, which had previously been shown to bind specifically to the active site of the enzyme. The peptide containing the bound nucleotide was shown to have the sequence:

-Glu-Ser-Gly-Ala-Gly-Lys-Thr-XXX-Asn-Thr-Me_2Lys-Lys-Val-Ile-Gln-Tyr

where XXX represents the residue to which the UTP was attached. This sequence is identical to a sequence in the myosin II gene where XXX is a Gln residue and is highly homologous with sequences in rabbit and nematode striated myosins (differing at only 2 positions) that are thought to be in the region of the ATP-binding site.

Last year Dr. Hammer reported the isolation of a myosin II heavy chain gene. He has now obtained the following additional information. The 3' end of the gene has been identified by showing that the amino acid sequence deduced from the nucleotide sequence of this region of the gene matched exactly the amino acid sequence of the C-terminal peptide, which had been sequenced by protein chemical methods. The 5' end of the gene has been approximately identified indicating that the myosin II genomic clone contains at least 90% of the coding information for the heavy chain and that the gene contains very little intron DNA. About 25% of the gene has been sequenced. Most of the sequence obtained is for the myosin head and shows $\sim$60% exact homology with other sequenced myosin heavy chains.

Acanthamoeba Myosin I: Myosins IA and IB from Acanthamoeba are structurally atypical myosins in that they contain only a single heavy chain and a single light chain and in that the heavy chains are much smaller (130 kDa and 125 kDa vs about 200 kDa for other myosins). Most importantly, myosins IA and IB are incapable of forming bipolar filaments that are generally thought to be essential for contractile activity of actomyosin complexes.

When myosin I ATPase activity is measured as a function of actin concentration, there is an initial activation phase, followed by an inactivation phase as the actin concentration is increased, and then a re-activation phase at still higher actin concentrations. Dr. Korn has now proposed a reason for this phenomenon which can form the basis of the mechanical functioning of myosin I in contractile events. Enzyme kinetic data support the idea that the myosin I heavy chain may possess two actin-binding sites: a high-affinity site that is ATP-insensitive and not directly involved in catalytic activity and a low-affinity, ATP-sensitive site that is the catalytic site.

This kinetic hypothesis predicts that myosin I should crosslink actin filaments. It has been possible to correlate actin-activated ATPase activity with superprecipitation (i.e. "contraction") of actin-myosin I filaments. Crosslinking of F-actin by myosin I occurred in the absence of ATP more strongly than in its presence and equally well for unphosphorylated and phosphorylated myosin I. But superprecipitation occurred only in the presence of MgATP and when the myosin was phosphorylated which is the only form of myosin I that shows actin-activated ATPase activity. These studies prove that a single heavy chain of 125-130 kDa can contain all of the essential structural elements for contractile activities and that, providing the heavy chain has two actin-binding sites, myosin filaments

are not required for actomyosin contraction to occur.

Last year, Dr. Hammer reported the isolation of a myosin IB heavy chain gene tentatively identified by hybrid selection analysis and Northern blotting. Dr. Hammer has now confirmed the identity of the clone by specific immunoprecipitation of protein translated from hybrid-selected mRNA. About 20% of the gene has been sequenced. The amino acid sequence so far deduced from the nucleic acid sequence shows that myosin IB is clearly related to typical sarcomeric myosins: the deduced amino acid sequences encompassing the putative ATP binding site and active thiol in the myosin IB protein (totally 250 amino acids) show 60% homology with typical sarcomeric myosin heavy chains).

Biochemistry of Muscle Contraction: Drs. Eisenberg and Greene's model of muscle contraction proposes that as ATP is hydrolyzed the attached myosin cross-bridge oscillates between the 45° strong-binding conformation and the 90° weak binding-conformation. There has been little information, however, as to the structure of the latter conformation, which occurs only transiently in the cross-bridge cycle when ATP or ADP·Pi is bound to myosin. By using S-1 covalently cross-linked to actin, they have now studied the structure of cross-linked actin·S-1 in the presence of ATP by electron microscopy. Cross-linked actin·S-1 has the key property of hydrolyzing ATP at a rate comparable to the maximal actin-activated ATPase of S-1, indicating that the cross-linked S-1 may undergo the conformational changes associated with the hydrolysis of ATP. In the absence of ATP, the appearance of the cross-linked filaments closely resembled the rigor appearance obtained using non-cross-linked proteins. The arrowheads had the conventional structure, and individual S-1 molecules were elongated, curved and appeared to make an angle of 45° with the thin filament. The addition of ATP to the cross-linked actin·S-1 complex caused a radical change in the structure of the cross-bridges. Individual S-1 molecules now appeared to be attached at variable angles which, in contrast to rigor, did not center on 45°. The cross-linked actin·S-1 preparation no longer showed the arrowhead pattern of S-1 decoration, but instead appeared disordered with little obvious polarity. It therefore appears that, in agreement with the model, states A·M·ATP and A·M·ADP·P_i have very different conformations from the classic arrowhead conformation of the A·M state.

The troponin-tropomyosin complex can also be used as a probe for the 45° and 90° conformations since this regulatory complex affects the two S-1 conformations very differently. The model predicts that when S-1 is cross-linked to actin it can oscillate between the 90° and 45° conformations, depending on the nucleotide bound to S-1. Based on Eisenberg and Greene's previous studies when pPDM-modified S-1 and NEM-modified S-1 are cross-linked to actin, the cross-linked actin·pPDM·S-1 and the cross-linked actin·NEM·S-1 should remain always in the 90° and 45° conformation, respectively, regardless of the nucleotide bound. The results showed that both in the presence of ADP and ATP, the crosslinked NEM·S-1 remains in the strong-binding conformation, while the crosslinked pPDM S-1 remains in the weak-binding conformation. In contrast, crosslinked unmodified S-1 is in the weak-binding conformation in the presence of ATP and the strong-binding conformation in the presence of ADP.

Eisenberg and Greene's detailed kinetic model for the hydrolysis of ATP by actomyosin suggests that binding of ATP to actomyosin weakens the binding of myosin to actin by more than four orders of magnitude. While myosin is in the weak binding conformation, ATP hydrolysis occurs at the active site and then a rate-limiting step occurs. Eisenberg and Greene have proposed that both the

ATP hydrolysis step and the rate-limiting step occur at about the same rate whether the myosin is bound to or dissociated from actin. Following the rate-limiting step, they propose that P_i release is rapid and is associated with a conversion of the myosin molecule back to the strong binding conformation.

The model can be tested by measuring the rate of O^{18} exchange during ATPase activity by both cross-linked acto-S-1 and non-cross-linked S-1 at varying concentrations of actin. The results suggest that much less O^{18} exchange occurs than would be predicted by the Eisenberg and Greene model, if actin had no effect on the freedom of rotation of Pi. Furthermore, the low level of exchange observed seems to be similar to the low level of exchange observed by other workers with myofibrils. The simplest explanation of the data may be that the binding of actin to S-1 reduces the freedom of rotation of the Pi at the active site both *in vivo* and *in vitro* when the weak binding state S-1·ADP·Pi^1 binds to actin. If this explanation is valid, it would be the first demonstration in any system that freedom of rotation of Pi, as opposed to the rate constants in a kinetic cycle, determine the amount of O^{18} exchange which occurs during ATP hydrolysis. It would also be consistent with Eisenberg and Greene's view that there is a special rate-limiting step in the acto-S-1 ATPase cycle. This step is of major importance in the physiology of muscle contraction because it determines the shape of the force-velocity curve.

A second major test of the model is to relate it to the physiological behavior of muscle fibers. A major prediction of the model is that cross-bridges in the weak-binding state will be in rapid equilibrium between attachment and detachment to actin. Eisenberg and Greene previously published evidence that this occurs in the presence of ATP with single skinned psoas muscle fibers. They have now obtained evidence that this same behavior occurs with the ATP analogs, AMPPNP and PPi, although the rates of attachment to and detachment from actin are slower.

Greene and Eisenberg had previously suggested that at low ionic strength, troponin-tropomyosin regulates the actomyosin ATPase activity by inhibiting a kinetic step in the actomyosin ATPase cycle rather than by blocking the binding of S-1 to actin. This leads to the prediction that troponin-tropomyosin should inhibit the ATPase activity of acto·S-1 even when S-1 is cross-linked to actin. With a cross-linked preparation containing low S-1 to actin ratios, troponin-tropoymosin caused 90-95% inhibition of the ATPase activity. Therefore, these results are in agreement with the suggestion that troponin-tropomyosin regulates primarily by inhibiting a kinetic step in the ATPase cycle.

Experiments were also done to test whether S-1·ATP, which binds to regulated actin with no apparent cooperativity, shifts the position of tropomyosin on the regulated actin filament. The model predicts that in the absence of Ca^{2+}, regulated actin is in the weak form and this form should not be shifted by the binding of S-1·ATP. The ATPase activity of regulated acto·S-1 was measured in the presence of varying concentrations of pPDM·S-1 to determine whether pPDM·S-1 could turn on the regulated actin filaments. In the absence of Ca^{2+}, when the regulated actin filament was 50% saturated with pPDM·S-1, the rate of the regulated acto·S-1 was only 5% of the maximal ATPase activity. This showed that pPDM·S-1 did not significantly shift the tropomyosin from its weak binding-inhibitory position on the actin filament. In contrast, in the presence of Ca^{2+}, pPDM·S-1 readily turns on the ATPase activity of regulated acto·S-1 to an extent comparable to that observed with NEM·S-1 which completely turn on regulated actin These results are consistent with the binding data which show that Ca^{2+} causes

a partial shift in the position of the tropomyosin, thus making it easier for S-1 binding to fully shift the tropomyosin into the strong binding state of regulated actin.

Microtubule Structure and Function:

MAP-2 is the most conspicuous protein (MW 270,000) in mammalian brain that co-assembles in constant proportion to tubulin, and promotes in vitro polymerization of pure tubulin. Dr. Flavin has previously found that MAP-2 isolated from assembly-cycle purified microtubule protein contains 10 mol phosphate/mol. This "A subset" of MAP-2 phosphate can only be phospho-labeled by turnover in living brain, as the phosphatase and kinase involved have not been identified. An additional 10 mol of phosphate, the "B subset", are added to MAP-2 by the cAMP-dependent kinase which copurifies with microtubules.

Last year Dr. Flavin reported preliminary evidence that B sites were in both microtubule-binding and projection domains, whereas A sites were confined to the projection domains. The B subset has now been localized more precisely, 6 are in the binding domain and 4 are in the 240-kDa projection domain; the A subset distributed 9.5 in the latter, and only 0.5 in the binding domain.

Using MAP-2 labeled in the B subset as assay substrate, Dr. Flavin has purified a phosphatase from brain to near homogeneity. PAGE analysis showed comparable amounts of 4 polypeptides, 3 of which corresponded in molecular weight to the subunits of smooth muscle phosphatase 1. Although it released label from the A subset relatively slowly, the enzyme could be used to release the bulk of these phosphatases, in contrast to the results reported last year for other protein phosphatases. It may be that this is the enzyme responsible for A subset turnover in vivo.

Bioenergetics: Last year Dr. Hendler found that cytochrome c_1 of beef heart mitochondria, previously thought to be a single species with an Em of 230 mV capable of transferring one electron, could be resolved with two thermodynamic species with Em values of 200 and 255 mV, each of which passes 2 electrons at a time. This year Dr. Hendler has completed a similar spectral and potentiometric analysis of cytochromes aa_3 in beef heart mitochondria. Three Nernstian components were found with E_m values of 200, 260, and 340 mV, n values of 2, 2, 1 (electrons passed), and maximum α absorbance at 602, 605, and 607 nm respectively. Using the fact that only cytochrome a_3 can bind carbon monoxide, Dr. Hendler identified the lowest E_m component as cytochrome a_3, contrary to the current view that cytochrome a_3 is the component with E_m 350 mV. The E_m of the carbon monoxide binding component was not raised in the carbon monoxide atmosphere although the Nernst equation predicts such a rise whenever a ligand binds more tightly to the reduced form of a redox couple. However, this result can be explained in terms of the Nernst equation if a Bohr-type effect occurs where protons are released upon reduction of the liganded form. The results are therefore consistent with current ideas that cytochrome oxidase is a proto-pumping enzyme.

In a separate study, purified cytochrome aa_3 was analyzed using a number of different experimental and analytical methods. The results obtained with the free enzyme were the same as obtained with the mitochondrial enzyme. The total approach defines three Nernstian components: (1) E_m 200 mV, n = 2, Soret peak at 429 nm and α peak at 602 nm. (2) E_m 260 mV, n = 2, Soret peak at 446 nm and α peak at 605 nm. (3) E_m 340 mV, n = 1, Soret peak at 448 nm and α peak at

607 nm. The spectrum of the low E_m component, which Dr. Hendler identifies as cytochrome a_3, is identical to that of an unstable form of cytochrome oxidase which has a higher turnover number than the resting enzyme and which has been called the "pulsed" or "oxygenated" form. Hendler's data show that no O_2 is present in this species, however, and he suggests that the spectrum is due to an unstable conformer of the reduced heme a_3 center. Other experiments indicate that the redox state of heme a controls the redox potential of heme a_3.

Hendler's consistent findings of n = 2 stages of electron tranport at all stages of the respiratory chain, namely cytochromes b, cytochromes c_1, and cytochromes aa_3, mark a major departure from the current view that the respiratory chain passes only one electron at a time. Hendler proposes that an n = 2 electron transport chain is compatible with the following considerations : (1) 2 electrons are removed from the substrate being oxidized. (2) The addition of a single electron to O_2 is an unlikely event because of the thermodynamic instability and high reactivity of superoxide (O_2). A 2 electron addition to O_2 would bypass the intermediate with the formation of a peroxaditic form of O_2 ($O_2^=$). (3) The cytochrome bc_1 segment of the respiratory chain is found to be present as a dimer. (4) The cytochrome aa_3 segment of the chain is normally found as a dimer.

The Mitchell hypothesis predicts certain fixed ratios for proton translocation per atom of O consumed and prohibits proton translocation at site III (cytochrome oxidase) because this segment of the chain has no known proton carrying member. Workers in this field are in sharp disagreement on whether 0, 2 or 4 protons are translocated at site III (cytochrome aa_3), i.e. 4, 6 or 8 for sites II and III.

Dr. Hendler has developed a new methodology, described last year, which allows direct acquisition of reliable data during the first second of a respiratory pulse. Succinate has been used as the electron donor through sites II and III in a suspension of intact rat liver mitochondria and reduced cytochrome c as the electron donor through site III in a suspension of rat liver mitoplasts. Both systems show an initial burst in H^+/O ratio which peaks within 50 ms to values of 20 to 30 for the succinate system and to about 10 for the cytochrome c system. The burst is completely damped at about 300 ms. This phenomenon has not been seen previously, because no one else has obtained data in such short time spans close to zero. Control experiments appear to have eliminated the possibility of artifacts and it seems most likely at this time that the burst is a real physiological phenomenon.

When Dr. Hendler analyzes his data in the conventional way, and using only data obtained after 1 s as other have necessarily done, he confirms Lehninger's value of 8 for H^+/O ratio for sites II plus III. However, Hendler does not believe this number is significant because to extrapolate to a ratio of 8 the real data obtained by Hendler between 0 and 1 second (which indicate a ratio of 20-30) must be ignored. It is Dr. Hendler's view that there may not be a fixed H^+/O ratio, which requires that the processes of O_2 consumption and H^+ translocation are directly linked in a specific way. Instead, the energy liberated by electron transport may be transferred to a proton pump which will pump the number of protons possible against the existing $\Delta\mu_{H+}$. The H^+/O ratio would then be a variable, very high at first when $\Delta\mu_H$ is near zero and rapidly dropping as $\Delta\mu_H$ builds.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00401-19 LCB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Potentiometric studies of respiratory components of E. coli and mitochondria

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard W. Hendler	Section Head	LCB, NHLBI
	K.V.S. Reddy	Visiting Fellow	LCB, NHLBI

COOPERATING UNITS *(if any)*

Richard I. Shrager, Mathematician, LAS, DCRT; Barry Bunow, Biomathematician, LAS, DCRT; John S. Rieske, Professor, Ohio State Univ.; Winslow S. Caughey, Dept. Chairman, Colorado State Univ.

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Membrane Enzymology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Using procedures developed in this laboratory for the acquistion and analysis of more data than is normally processed for spectral potentiometric studies, we have completed the analysis of cytochrome aa-3 in intact mitochondria and in isolation. Our analyses support the following unique new characteristics for the enzyme. (1) There are three Nernstian components. (2) The Em values are 200 mV, 260 mV, and 340 mV with corresponding n values of 2, 2, and 1. (3) The individual absorption maxima for the Soret and α peaks are 429 and 602 nm; 446, and 605 nm; and 448 and 607 nm, respectively. (4) Cytochrome a-3 is the component with Soret maximum of 429 nm and α at 602 nm. (5) The redox potential of heme a-3 is under the control of the redox state of heme a; it is 200 mV when heme a is reduced and > 420 mV when heme a is oxidized. (6) The Em of heme a-3 is not raised in an atmosphere of carbon monoxide and this behavior indicates that protons are held less strongly by the reduced liganded enzyme than the oxidized form.

The general result of our studies with all of the mitochondrial cytochromes is that the electron transport chain passes 2 electrons at a time rather than one. This conclusion is consistent with a number of other observations. We have analyzed earlier methods of data acquistion and analysis and have found that they are not capable of resolving the mixture and character of cytochromes revealed by our methods.

PHS 6040 (Rev. 1/84) GPO 914-918

Methods Employed and Major Findings

The spectral and potentiometric analysis of cytochromes aa_3 in beef heart mitochondria was completed. Three Nernstian components were found with E_m values of 200, 260, and 340 mV, n values of 2, 2, 1; and maximum α absorbance at 602, 605, and 607 nm respectively. Using the fact that only cytochrome a_3 can bind carbon monoxide, we have performed titrations under a carbon monoxide atmosphere to identify this heme center. Under these conditions, only the species with E_m 200 mV, and α peak at 602 nm was effected. This species disappeared and was replaced by a species with an α peak at 594 nm, characteristic of the cytochrome a_3 - CO adduct. The results of these studies differ markedly from earlier studies based on the use of a 2-point ΔA method of data collection and analysis by the procedures of Wilson and Dutton. The existing picture based on these techniques is that only two Nernstian components are present, with E_m values of 230 and 350 mV, both with n values of 1. Whereas, we have identified the lowest E_m component as cytochrome a_3 the current view is that cytochrome a_3 is the component with E_m 350 mV. A further important difference in our work is that the E_m of the carbon monoxide binding component was not raised in the carbon monoxide atmosphere. The Nernst equation predicts such a rise whenever a ligand binds more tightly to the reduced form of a redox couple, and it is commonly accepted that this rise occurs. We have found that the 594 nm component (i.e. CO-adduct) has the same E_m as the 602 nm (i.e. unliganded) component. This result can be explained in terms of the Nernst equation if a Bohr-type effect occurs where protons are released upon reduction of the liganded form. Our results are therefore consistent with current ideas that cytochrome oxidase is a proton-pumping enzyme. In a separate study, purified cytochrome aa_3 was analyzed. The pure enzyme allowed the use of the Soret absorption region (400-500 nm) as well as the α region (600-700 nm). For these studies a new electrical titration system was developed. In this system known voltages are set and held on the working platinum electrode to avoid possile damage to the pure enzyme by excessive voltages that may develop in the usual constant current system used with mitochondria. The results obtained with the free enzyme were the same when either the newer or older electrical system was used and when chemical titrations were performed instead of electrical titrations. The results obtained with the free enzyme when the α absorption region was used were the same as obtained with the mitochondrial enzyme. The results obtained using the Soret region data with the pure enzyme were the same as obtained with the α region data and the composite picture obtained by separate analyses of these regions was independently arrived at by the SVD procedure which uses both regions. The total approach defines three Nernstian components: (1) E_m 200 mV, n = 2, Soret peak at 429 nm and α peak at 602 nm. (2) E_m 260 mV, n = 2, Soret peak at 446 nm and α peak at 605 nm. (3) E_m 340 mV, n = 1, Soret peak at 448 nm and α peak at 607 nm. Under the carbon monoxide atmosphere, in addition to the CO-adduct α peak at 594 nm, the pure enzyme allowed the analysis of the CO-adduct Soret peak at 435 nm. This analysis confirmed the finding based on the α peak in both mitochondria and pure enzyme, that the E_m of the CO-liganded species was not raised. The low E_m component which we have identified as cytochrome a_3 has a spectrum with a Soret at 429 and an α peak at 602, which is identical to an unstable form of cytochrome oxidase which has a higher turnover number than the resting enzyme and which has been called the "pulsed" or "oxygenated" form. Chance and collaborators believe that this form of the enzyme has a peroxide form of O_2 present. We are certain that no form of O_2 is present in our species and believe that the spectrum is due to an un-

stable conformer of the reduced heme a_3 center. A finding of major importance is that the 429 species is seen at low voltages (i.e. 200 mV) only when the heme a centers are reduced. When the heme a centers are oxidized, a reductive titration of the heme a causes an oxidation of the 429 nm species. This indicates that the E_m of heme a_3 is above that of heme a when heme a is oxidized and rapidly drops to below that of heme a when the heme a is reduced. In other words, the redox state of heme a controls the redox potential of heme a_3. Our consistent findings of n = 2 stages of electron tranport at all stages of the respiratory chain, namely cytochromes b (JBC 258, 8568 (1983), cytochromes c_1 (submitted)), and cytochromes aa_3 (submitted) mark a major departure from the current view that the respiratory chain passes only one electron at a time. The one electron chain concept is based on methods of data acquistion and analysis in wide use during the past 15 years. In a comprehensive analysis of these methods we have been able to show that these techniques are inadequate and not capable of resolving the mixture of redox components which each of our two newer methods of analysis has resolved. An n = 2 electron transport chain is compatible with the following considerations : (1) 2 electrons are removed from the substrate being oxidized. (2) The addition of a single electron to O_2 is an unlikely event because of the thermodynamic instability and high reactivity of superoxide (O_2^-). A 2 electron addition to O_2 would bypass the intermediate with the formation of a peroxaditic form of O_2 ($O_2^=$). (3) The cytochrome bc_1 segment of the respiratory chain is found to be present as a dimer. (4) The cytochrome aa_3 segment of the chain is normally found as a dimer.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00409-15 LCB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interaction of Actin and Myosin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Evan Eisenberg	Section Head	LCB, NHLBI
Others:	John A. Evans	Staff Fellow	LCB, NHLBI
	Lois E. Greene	Research Chemist	LCB, NHLBI
	Jose Biosca	Visiting Fellow	LCB, NHLBI

COOPERATING UNITS *(if any)*

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Laboratory of Cell Biology

SECTION

Section on Cellular Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2 2/3	2 2/3	0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

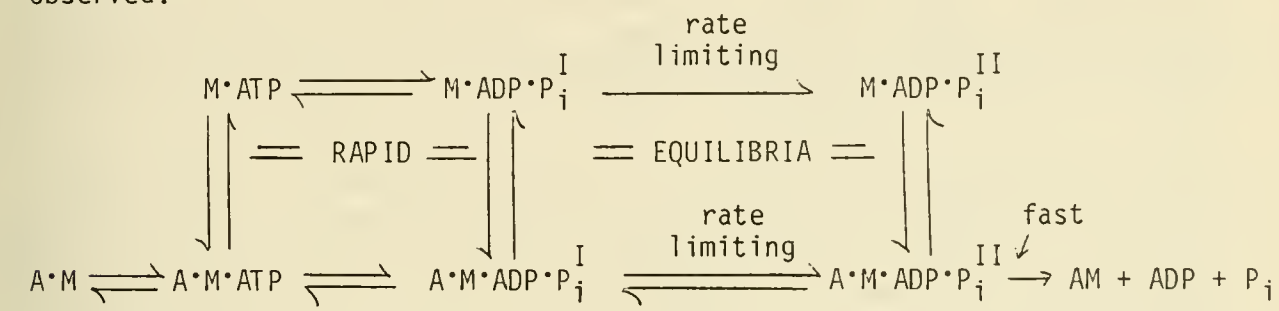
SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have proposed a cross-bridge model of muscle contraction in which, during each cycle of ATP hydrolysis, the myosin cross-bridge alternates between a conformation in which the cross-bridge binds strongly to actin, and a conformation in which the cross-bridge binds weakly to actin with a rapid equilibrium existing between the attached and detached cross-bridge. While the cross-bridge is in the latter conformation the hydrolysis step and a separate rate limiting step occur. In the present study, we have tested several predictions of this model both in vitro and in single skinned rabbit muscle fibers. First, we measured O-18 exchange with S-1 chemically cross-linked to actin and found that much less O-18 exchange occurs than is predicted by our kinetic model. Yet, as predicted by our model, ATP hydrolysis does not appear to be the rate-limiting step in the ATPase cycle. Together these results suggest that the binding of actin to S-1 may affect the freedom of the rotation of Pi at the active site of S-1. Since such an effect on O-18 exchange has not previously been observed, it may have major implications for the mechanism of O-18 exchange occurring both in vitro and in vivo in muscle and also in other systems such as mitochondria. We also studied the effect of AMPPNP and PPi on relaxation of force in single muscle fibers. Among other effects, our results suggested that AMPPNP and PPi show much weaker binding to actin bound cross-bridges than was previously observed by other workers. We therefore reinvestigated the binding of AMPPNP and PPi to acto·S-1 in vitro and here too we found much weaker binding than was previously reported. This result is important in interpreting the numerous structural and mechanical studies carried out with these ATP analogues on muscle fibers.

Project Description:

Objectives: The accepted mechanism for the contraction of muscle in vivo involves the interaction of actin and myosin filaments. However, the exact nature of the energy transduction mechanism by which the energy stored in the ATP molecule (relative to ADP and P_i) is converted to useful work is not well understood. By studying the biochemical interaction of actin and myosin in vitro we hope to gain insight into the in vivo energy transduction mechanism.

We have proposed the following model for the actin-activated myosin subfragment-1 (S-1) ATPase activity, based on pre-steady-state and steady-state studies of the binding of S-1 to actin in the presence of ATP, and the fact that no significant inhibition of the actin-activated ATPase by actin has ever been observed:



where M = myosin and A = actin. This model suggests that, after ATP binds to actomyosin, the binding of myosin to actin becomes weakened by more than four orders of magnitude. While myosin is in the weak binding conformation, ATP hydrolysis occurs at the active site and then a rate-limiting step occurs. We have proposed that both the ATP hydrolysis step and the rate-limiting step occur at about the same rate whether the myosin is bound to or dissociated from actin. Following the rate-limiting step, we have proposed that P_i release is rapid and is associated with a conversion of the myosin molecule back to the strong binding conformation.

One aspect of this model, about which there is still disagreement, is the nature of the rate-limiting step. It has been proposed that the rate-limiting step is the transition from A·M·ATP to A·M·ADP·P_i rather than the subsequent conformational change from A·M·ADP·P_i^I to A·M·ADP·P_i^{II}. Another question of importance is to determine whether the behavior predicted by this model occurs in single rabbit muscle fibers.

Methods Employed and Major Findings: We have continued our investigation of the rate of O^{18} exchange during ATPase activity by both cross-linked acto-S-1 and non-cross-linked S-1 at varying concentrations of actin. Our results suggest that, under a wide variety of conditions, much less O^{18} exchange occurs than would be predicted by the 6-state model, if actin had no effect on the freedom of rotation of Pi. Furthermore, the low level of exchange observed seems to be similar to the low level of exchange observed by other workers with myofibrils. Here too no simple kinetic explanation has been provided to explain the low level of O^{18} exchange. Since our data continue to indicate that the ATP hydrolysis step is not the rate-limiting step on the ATPase cycle, i.e. that the

above kinetic model is valid, we think that the simplest explanation of our data is that the binding of actin to S-1 reduces the freedom of rotation of the Pi at the active site both *in vivo* and *in vitro* when the weak binding state S-1·ADP·Pi^I binds to actin. Hence, although forward and reverse transitions between acto-S-1·ATP and acto-S-1·ADP·Pi occur a number of times before the rate limiting step and product release occur, there is no O^{18} exchange exchange because the Pi is not free to rotate. If this explanation is indeed valid, it would be the first demonstration in any system that freedom of rotation of Pi, as opposed to the rate constants in a kinetic cycle, determine the amount of O^{18} exchange which occurs during ATP hydrolysis. It would also be consistent with our view that there is a special rate-limiting step in the acto-S-1 ATPase cycle. This step is of major importance in the physiology of muscle contraction because it determines the shape of the force-velocity curve.

A second major area of research in testing our model is to relate it to the physiological behavior of muscle fibers. A major prediction of our model is that cross-bridges in the weak-binding state will be in rapid equilibrium between attachment and detachment to actin. We previously published evidence that this occurs in the presence of ATP with single skinned psoas muscle fibers. We have now obtained evidence that this same behavior occurs with the ATP analogs, AMPPNP and PPi, although the rates of attachment to and detachment from actin are slower. In the course of these experiments, we observed that the apparent binding constant of AMPPNP and PPi to the cross-bridges *in vivo* appeared to be much weaker than had previously been proposed. We therefore reinvestigated the binding of AMPPNP and PPi to acto-S-1 *in vitro* using both direct binding studies and measurements of K_I, the ability of AMPPNP and PPi to inhibit the acto-S-1 ATPase activity. The results obtained with both S-1 cross-linked to actin and S-1 studied at high actin concentration all show much weaker binding of AMPPNP and PPi than had previously been suspected. This suggests that many of the structural and mechanical studies carried out on muscle fibers must be reinterpreted since even at 4 mM AMPPNP, the cross-bridges may only 1/3 to 1/2 saturated wtih nucleotide.

Since our values for the binding of AMP-PNP and PPi to acto·S-1 disagree with the results of earlier work on single rabbit psoas fibers, we are presently extending our experiments to studies on myofibrils. We have learned to prepare myofibrils from rabbit psoas fibers with different amounts of overlap between the actin and myosin filaments. We are presently carrying out binding studies with these myofibrils which should show very different amounts of bound nucleotide depending on the amount of overlap between the actin and myosin filament.

Significance to Biomedical Research: Understanding the mechanism of the actomyosin ATPase is central to gaining an understanding of muscle contraction as well as many other motile systems. This knowledge, in turn, may have important applications in the study of muscular dystrophy and heart disease.

Proposed Course of Research: During the next year, we plan to continue our investigation of O^{18} exchange under a variety of conditions. At the same time we plan to use our new quench-flow and stopped-flow apparatus to confirm our crucial observation that ATP hydrolysis is not the rate-limiting step in the ATPase cycle when S-1 is bound to actin. Together these two findings will confirm first, that there is a special rate limiting step following the ATP hydrolysis step and second that bound actin does indeed affect the freedom of

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rotation of Pi at the active site of S-1. We also plan to continue our physiological studies on the effect of AMPPNP, PPi and ADP on relaxation of force in single rabbit psoas fibers and relate the results of these studies to biochemical studies on myofibrils with varying amounts of overlap between the actin and myosin filaments.

Publications:

Chalovich, J., Stein, L.A. Greene, L.E. and Eisenberg, E.: Interaction of Isozymes of myosin subfragment 1 with actin: Effect of ionic strength and nucleotide. Biochemistry 23: 4885-4889 (1984)

Stein, L.A., Greene, L.E., Chock, P.B. and Eisenberg, B.: Rate limiting step in the actomyosin adenosinetriphosphate cycle: Studies with myosin subfragment-one cross-linked to actin. Biochemistry 24: 1357-1363 (1985)

Eisenberg, E. and Hill, T.L.: Muscle contraction and free energy transduction in biological systems. Science 227: 999-1006 (1985)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00413-09 LCB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of regulation of the actomyosin ATPase

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Lois E. Greene	Research Chemist	LCB, NHLBI
Others:	David L. Williams, Jr.	Staff Fellow	LCB, NHLBI
	James R. Sellers	Staff Fellow	LMB, NHLBI
	Robert King	Summer Worker	Duke Univ.
	Evan Eisenberg	Section Head	LCB, NHLBI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1 2/3	1 2/3	1/6

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In our model of regulation, tropomyosin can exist in two positions on the thin filament, forming either the weak-binding state or the strong-binding state of regulated actin. In the weak-binding state, which occurs in the absence of Ca^{2+}, troponin-tropomyosin inhibits the binding of S-1 and S-1·ADP to actin, but not S-1·ATP. Therefore, ATPase inhibition seems to be due to regulated actin in the weak-binding state inhibiting Pi release. First, results were obtained consistent with the latter part of our model using S-1 which was cross-linked to actin. Troponin-tropomyosin was found to markedly inhibit the ATPase activity of cross-linked actin·S-1 both at low and high ionic strength. Since cross-linked S-1 appears to behave kinetically like S-1 in the presence of infinite actin concentration, the extensive regulation obtained with cross-linked S-1 is not due to troponin-tropomyosin blocking the binding of cross-linked S-1 to actin. Second, we examined the ability of pPDM·S-1, which is a stable analog of S-1·ATP, to turn on or potentiate the ATPase activity of regulated acto·S-1. Consistent with our binding data, which shows no apparent cooperativity in the binding of S-1·ATP or pPDM·S-1·ATP to regulated actin, pPDM·S-1·ATP does not significantly turn on the ATPase activity of regulated acto·S-1. These results indicate that tropomyosin remains in the inhibitory position when pPDM·S-1·ATP binds to regulated actin. Third, we found that in contrast to the results obtained with skeletal muscle, the inhibition of P_i release in the actomyosin ATPase cycle of smooth muscle is not accompanied by inhibition of the binding of myosin·ADP to actin. These results show that although inhibition of Pi release appears to be a general mechanism of muscle regulation, it is not always coupled to inhibition in the binding of myosin·ADP to actin.

Objectives: In our model of muscle regulation, troponin-tropomyosin inhibits the actomyosin ATPase by blocking a kinetic step in the ATPase cycle rather than by blocking the binding of S-1 to actin. This leads to the prediction that troponin-tropomyosin should inhibit the ATPase activity of acto·S-1 even when S-1 is cross-linked to actin. This was examined using S-1 cross-linked to actin by ethyl-3[3-(dimethylamino)proply] carbodiimide. Another prediction of our model which we tested is that S-1·ATP, which binds with no apparent cooperativity to regulated actin, should not cause a shift in the position of tropomyosin on the regulated actin filament. This was tested using pPDM·S-1·ATP, an analog of S-1·ATP, by determining whether pPDM·S-1 could turn on the regulated actin filament, i.e. shift it to the strong form. Another aspect of our model is that with skeletal muscle, inhibition of the Pi release step in the actomyosin ATPase cycle appears to be coupled to the weak binding of S-1·ADP to regulated actin. In smooth muscle, relaxation is also caused by inhibition of Pi release, but here phosphorylation of myosin, rather than troponin-tropomyosin, controls the regulation. We were interested in determining whether with smooth muscle too, the inhibition of the Pi release step in the actomyosin ATPase cycle is correlated with the weak binding of HMM·ADP to actin.

Major Findings and Methods Employed: We had previously suggested that at low ionic strength, troponin-tropomyosin regulates the actomyosin ATPase activity by inhibiting a kinetic step in the actomyosin ATPase cycle rather than by blocking the binding of S-1 to actin. This leads to the prediction that troponin-tropomyosin should inhibit the ATPase activity of acto·S-1 even when S-1 is cross-linked to actin. This was tested by using S-1 cross-linked to actin by the zero length cross-linker, 1-ethyl-3-[3-dimethylamino]propyl-carbodiimide, in which the cross-linked filament had a ratio of one S-1 to five actin monomers. Troponin-tropomyosin only inhibited by 70% the ATPase activity of this cross-linked preparation, whereas it caused 95% inhibition of the ATPase activity of non-cross-linked proteins. However, if we used a cross-linked preparation containing low S-1 to actin ratios made under mild cross-linking conditions, troponin-tropoymosin caused 90% inhibition of its ATPase activity. At μ = 18mM, 25° C, the ATPase activity of this latter cross-linked preparation is only about 2-fold greater than the maximal actin-activated ATPase activity of S-1 obtained with regulated actin in the absence of Ca^{2+}. At physiological ionic strength the ATPase activity of this cross-linked S-1-actin preparation is inhibited about 95% by troponin-tropomyosin. Since cross-linked S-1 behaves kinetically like S-1 in the presence of infinite actin concentration, inhibition of the ATPase activity of cross-linked actin-S-1 is not due to blocking of the binding of S-1 to actin. Therefore, these results are in agreement with the suggestion that troponin-tropomyosin regulates primarily by inhibiting a kinetic step in the ATPase cycle.

We also examined whether S-1·ATP, which binds to regulated actin with no apparent cooperativity, shifts the position of tropomyosin on the regulated actin filament. Our model predicts that in the absence of Ca^{2+}, regulated actin is in the weak form and this form should not be shifted by the binding of S-1·ATP. We tested this using pPDM·S-1, an analog of S-1·ATP, by determining whether pPDM·S-1 could turn on the regulated actin filament. The ATPase activity of regulated acto·S-1 was measured in the presence of varying concentrations of pPDM·S-1. To obtain the maximal turned on rate, the ATPase activity of regulated acto·S-1 was measured in the presence of NEM·S-1, which completely turns on the regulated actin. We found that in the absence of Ca^{2+}, when the regulated actin filament

was 50% saturated with pPDM·S-1, the rate of the regulated acto·S-1 was only 5% of the maximal ATPase activity. This showed that pPDM·S-1 did not significantly shift the tropomyosin from its weak binding-inhibitory position on the actin filament. In contrast, in the presence of Ca^{2+}, pPDM·S-1 readily turns on the ATPase activity of regulated acto·S-1 to an extent comparable to that observed with NEM·S-1. These results are consistent with our binding data which show that Ca^{2+} causes a partial shift in the position of the tropomyosin, thus making it easier for S-1 binding to fully shift the tropomyosin into the strong binding state of regulated actin. In addition, these results suggest that pPDM·S-1 must bind only slightly better to the strong binding state than to the weak binding state of regulated actin to account for the turning on of the ATPase activity in the presence of Ca^{2+}, but at the same time, the apparent lack of cooperativity in its binding to regulated actin.

We also examined the effect of phosphorylation on the binding of smooth muscle myosin·ADP to actin. Relaxation of both smooth and skeletal muscles appears to be caused primarily by inhibition of a kinetic step in the actomyosin ATPase cycle, the step associated with P_i release, rather than by a block in the binding of the myosin·ATP and myosin·ADP·Pi complexes to actin. In skeletal muscle, troponin-tropomyosin not only causes marked inhibition of P_i release, but it also inhibits the binding of S-1·ADP to actin, raising the possibility that the two phenomena are coupled in some way. We wanted to determine whether dephosphorylation of smooth muscle HMM also affects both the P_i release step and the binding of HMM·ADP to actin. This was done by competing phosphorylated and nonphosphorylated HMM for sites on F-actin. In contrast to skeletal muscle, the binding of smooth HMM to actin in the presence of ADP was not significantly inhibited as part of the regulatory process. At μ = 30 mM, dephosphorylation only decreases by $\sim$4 fold the affinity of each head of HMM for actin ($\sim$12-fold for the molecule), while at μ = 170 mM, there was less than a 2-fold decrease in the affinity of each head. At both ionic strengths, there is marked inhibitio of the actin-activated ATPase activity of smooth HMM by dephosphorylation. Therefore, the inhibition of P_i release with smooth acto·HMM, which could be as large as 900-fold, is not accompanied by inhibition of the binding of myosin·ADP to actin. We have also found that dephorylation decreases by only 3-fold the rate of P_i release from HMM alone. These results suggest that in smooth muscle, dephosphorylation inhibits the step associated with the release of P_i both in the forward and the reverse direction and this inhibition occurs without a corresponding effect on the binding of M·ADP to actin.

Significance to Biomedical Research: A number of studies have suggested that skeletal, cardiac, and smooth muscle, all have the same basic mechanism of muscle regulation. In addition, this mechanism of regulation of the actomyosin ATPase cycle may also occur in platelet, thymus, and microvilli. Since this mechanism of regulation is so wide-spread, it is especially important to understand it in detail.

Future work: We will examine the extent to which S-1·ATP turns on the ATPase activity of regulated acto·S-1. S-1·ATP, like pPDM·S-1·ATP, binds with no apparent cooperativity to regulated actin. We would therefore predict that S-1·ATP should not signigicantly turn on the ATPase activity of regulated actin in the absence of Ca^{2+}, although there have been reports by other workers that it does so. We will also examine the extent to which HMM, the two-headed fragment of myosin, turns on the ATPase activity of regulated acto·S-1. It has

previously been shown that when HMM has its full compliment of light chains, its binding to regulated actin is the presence of ATP shows Ca^{2+} sensitivity, unlike S-1. We want to determine whether this in turns indicates that HMM more readily turns on the ATPase activity of regulated actin than does S-1·ATP. We will also modify HMM with pPDM and measure its binding to actin both in the presence and absence of troponin-tropomyosin. This is to determine whether pPDM·HMM resembles HMM+ATP in its interaction with actin. We will also continue our kinetic studies on regulation by measuring the regulated actin-activated ATPase activity of S-1 both in the presence and absence of Ca^{2+}. This will be done under conditions in which the ATPase activity is fully turned off and fully turned on.

Publications:

Williams, D.L, Jr., Greene, L.E., Eisenberg, E.: Comparison of Effects of Smooth and Skeletal Muscle Tropomyosins on Interactions of Actin and Myosin Subfragment 1. Biochemistry. 23, 4150-4155, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00418-05 LCB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Electrochemical Potentials of Protons in Energy-Transducing Membranes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard W.Hendler	Section Head	LCB, NHLBI
	Oruganti Setty	Visiting Associate	LCB, NHLBI
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COOPERATING UNITS *(if any)*

Richard I. Shrager, Mathematician, LAS, DCRT: Barry Bunow, Biomathematician, LAS, DCRT; Baltazar Raynafare, Research Associate, Johns Hopkins Univ.; Albert Lehninger, Professor, Johns Hopkins Univ.; Jeffrey Froehlich, Medical Officer, NIA, NIH.

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Membrane Enzymology

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new system for direct determination of H+/O stoichiometric ratios during the rapid initiation of a respiratory pulse (developed in this laboratory) was used for extensive studies with two systems. Succinate as electron donor with intact mitochondria activated energy transduction sites II and III. Reduced cytochrome c and mitoplasts isolated site III. In both cases a new phenomenon was observed - a rapid burst in H+/O ratio peaking within 50 ms and dissipating by 300 ms. When data from 0 to 0.8 s was ignored and the remaining data extrapolated back to zero time on the assumption of 1st order kinetics, we confirmed Lehninger's zero time value of 8 for the succinate system as opposed to 6 favored by Wikstrom or 4 favored by Mitchell. However, our further analysis of the extrapolation procedure leads us to conclude that no value obtained by this technique is dependable. We are considering an alternative model for linkage between respiration and proton translocation other than the direct movement of one proton per electron as postulated in the Mitchell hypothesis. In this model there is no fixed ratio for H+/O, but rather the number would be a variable determined solely by the amount of energy liberated by respiration and the number of protons that could be moved against the $\Delta\mu H^+$ existing at the instant.

Work has continued on the completion and testing of an alternative experimental system for determining early H+/O. The new system does not employ relaxation time corrections for electrodes nor the presence of a carbon monoxide atmosphere.

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Methods Employed and Major Findings

The Mitchell hypothesis for the utilization of respiratory energy to form ATP can be viewed as having two parts. The first establishes that an electrochemical potential for protons ($\Delta\mu_{H+}$) is an important energy form in the process. The second part says that the $\Delta\mu_{H+}$ is formed directly from the respiratory chain by respiratory carriers which transfer the proton with the electron. This concept predicts certain fixed ratios for proton translocation per atom of O consumed and prohibits proton translocation at site III (cytochrome oxidase) because this segment of the chain has no known proton carrying member. If the actual H^+/O stoichiometry is not consistent with the Mitchell-type predictions, it means either that the respiratory chain is much different than currently viewed or that the means of forming $\Delta\mu_{H+}$ is other than the direct translocation of protons along wtih electrons in the chain. The latter possibility opens the question as to the manner of transferring respiratory energy to a separate device which pumps protons. Workers in this field have not raised the latter question but are in sharp disagreement on the number and location of Mitchell-style proton translocation "loops". The leading laboratories are divided into 3 groups. Everyone agrees that 4 protons are translocated per pair of electrons traversing site II (cytochrome bc_1 segment). At site III (cytochrome aa_3), Mitchell's followers say no protons, Wikstrom's followers say two protons and Lehninger's followers say four protons are translocated per pair of electrons. This controversy which has lasted nearly twenty years has become particularly heated during the past seven years. Our entry into the controversy has been based on the development of new methodology which for the first time allows direct acquisition of data during the first second of a respiratory pulse and which corrects for inherent delays in the response of electrode probes used in the assay. The system, described in the last report sharply initiates respiration upon photolysis of carbon monoxide-inhibited cytochrome oxidase. We have completed two separate studies with this system. In one, succinate was the electron donor through sites II and III in a suspension of intact rat liver mitochondria. In the other, reduced cytochrome c was the electron donor through site III in a suspension of rat liver mitoplasts. This preparation has the outer membrane stripped from the mitochondria allowing access of the protein electron donor to the inner membrane. Both systems show an initial burst in H^+/O ratio which peaks within 50 ms to values of 20 to 30 for the succinate system and to about 10 for the cytochrome c system. The burst is completely damped at about 300 ms. This phenomenon has not been seen previously, but no one else has obtained data in such short time spans close to zero. Individual records from the pH and O_2 electrodes show that both an enhanced rate of proton release and an initial lag in O_2 consumption figure in to the burst in H^+/O ratio. We have devoted considerable effort in exploring possibilities that the high values may be due in part to artifacts of measurements or to our correction procedures for electrode relaxation. Kinetic delays in the sensing of oxygen disappearance by the electrode in the bulk phase may have been caused by occluded oxygen pools in the mitochondrial matrix or in the layers of sintered glass covering the oxygen electrode. These effects were eliminated by suitable calculations. A variety of other assays were performed to show that the burst could not be attributed to the electrode correction procedures per se. That a slower rate of diffusion of O_2 relative to H^+ through an unstirred layer surrounding the mitochondria might have caused a lag in the two measurements was also evaluated and shown to be of no concern. That the burst is a real physiological phenomenon seems to be the most likely possibility

at this time.

During the past year the Lehninger laboratory has come under a sharp attack by Wikstrom's group in the form of a published critique. A case is made that Lehninger's value of 8 for the H^+/O ratio for combined sites II and III is an overestimate due to certain experimental defects, namely: (1) The oxygen electrode must first recover from tracking the rise of $[O_2]$ resulting from the injection of aerated buffer used to initiate respiration, before it can turn around and measure the O_2 consumption rate. Using a mathematical simulation, it was shown that this results in an overestiamte of H^+/O ratio. (2) Lehninger's technique of analyzing increments of $\Delta H/\Delta t$ and $\Delta O/\Delta t$ vs time to obtain initial dH/dt and dO/dt values, rather then analyzing [H] vs t and [O] vs t in order to obtain 1st order constants from which the derivatives can be computed, leads to significant errors. (3) The response time of Lehninger's electrodes introduce significant errors in the early data.

At Lehninger's request, Hendler and Shrager have drafted the major arguements, to be used in the form of a publication, to rebut Wikstrom et al.'s criticisms. We have been able to show that the model used by Wikstrom is faulty both in formulation and in mathematical development. Furthermore, in our approach which is free from the turnaround of the oxygen electrode; does not use rate data for analysis; and does consider electrode response times, we are able to confirm the Lehninger value of 8.

The most important part of this story, however, is not evident in our ability to confirm the Lehninger value of 8 vs the Wikstrom value of 6 or the Mitchell value of 4. We see our value of 8 as a confirmation of the Lehninger experimental number, but we do not share with him his belief in the "mecnanistic" or thermodynamic significance of the number. The number is arrived at by ignoring data from 0 to 0.8 s and by fitting the later data to a single exponential function of the type $[H^+]_t = A_H e^{-k_H t} + A_\infty$. The fitted equation is then back-extrapolated to zero time in order to obtain initial dH/dt. A similar process is followed for initial dO/dt and the ratio of these two values is taken as the zero time H^+/O value. Although both sets of data are readily fit to single exponential expressions, there is no theoretical or experimental support that either is single exponential. In fact, our analysis shows that the H^+ data is a composite of at least two and most likely more rate processes. The experimental model for which the H^+/O ratio is sought dictates that a fixed whole number of protons are translocated for each electron accepted by O. The only rate involved is dO/dt, and $dH/dt = n\, dO/dt$ where n is the stoichiometry. Therefore if the H^+ curve is single exponential, than the value of k in the exponential should be equal to the value of k in the oxygen equation. But it is found that k_H is significantly higher than k_O. This is readily explained by the fact that translocated protons leak back under the pull of the $\Delta\mu_{H+}$. The kinetics of the leak process lead to a distortion of the H^+ vs time curve so that a fit of the data using only a single exponential causes an increase in the value of k and also an increase in the value of the H^+/O ratio obtained by the back-extrapolation process. Another example that the H^+ data is not single exponential is the fact that when different ranges of the data are fit to the single exponential function, different values of k are obtained and H^+/O ratios which vary through the range of 6 to 10. A true one exponential curve should yield the same value of k regardless of the range used. We have also shown the weakness of this approach by using partially simu-

lated data. The k obtained by fitting the O_2 data was applied to an H^+ curve with a coefficient that yields a zero time H^+/O ratio of 6.0. The simulated H^+ curve was fit to actual H^+ data in order to obtain the best kinetic parameters for the leak process which is occurring. This composite curve which closely approximates the actual H^+ data was then fit with the single exponential function through different ranges of the data. As with the original data. different values of k and extrapolated H^+/O ratios in the range of 6 to 10, were obtained even though the underlying fixed H^+/O ratio for the two linked processes was set at 6.0. It is therefore apparent that any number obtained by these procedures is not to be trusted. In our view, there may not even be a fixed H^+/O ratio for the processes. A fixed number assumes that the processes of O_2 consumption and H^+ translocation are directly linked in a certain way. This concept, advanced by Mitchell has not been challenged, although there is no compelling data on which it is based. An alternative is that the energy liberated by electron transport is transferred to a proton pump which will pump the number of protons possible against the existing $\Delta\mu_{H+}$. The H^+/O ratio would then be a variable, very high at first when $\Delta\mu_H$ is near zero and rapidly dropping as $\Delta\mu_H$ builds. A more detailed consideration of the acquired data (not discussed here) leads us to favor this process as a viable alternative to the current view.

Work has been continuing on the development of an alternative technique to measure early H^+/O ratios. This approach which employs rapid mixing and flow techniques is in collaboration with Dr. Froehlich at NIA, NIH.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

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PERIOD COVERED
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TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure-function Relationships in Eukaryotic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Blair Bowers	Research Biologist	LCB, NHLBI
Others:	Tom Olszewski	Biologist	LCB, NHLBI
	Enrico Cabib	Sr. Research Chemist	LBM, NIADDKD
	Kyung Nam	Biologist	LCB, NHLBI

COOPERATING UNITS *(if any)*

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LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	1.0	1.6

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The morphology of short actin filament interaction with the myosin I from Acanthamoeba was examined. Short actin filaments were produced by addition of gelsolin to actin in a ratio of 1:50. With an actin/myosin I molar ratio of 10/1 slender bundles of actin formed and were stable in the absence of ATP. Actin filaments were held in parallel alignment with a spacing of about 69 nm. This was in contrast to controls, lacking myosin I, in which actin filaments often appear to be aligned with no space between them. The distance between two actin filaments in the bundles is compatible with a single myosin I molecule forming the cross-link. In two-dimensional arrays of cross-linked filaments the cross links appear to be spaced at 25-30 nm intervals along the filament. The three-dimensional appearance of actin (in the absence of gelsolin) and myosin I gels is that of an anastomosing network of long actin filaments that appear to be linked by association into bundles similar in appearance to those seen with short actin.

Project Description:

Objectives: These studies, conducted on two different eukaryotic systems, are in each case directed toward understanding the control mechanisms of a cellular event that has a morpholigically identifiable end point. We are examining factors that modulate endo- and exocytosis in Acanthamoeba, and attempting to trace membrane movements associated with these events. In other studies (in collaboration with E. Cabib, NIADDKD) the long range objectives are to elucidate the way in which yeast chitin synthetase, an enzyme found generally on the plasma membrane, can be selectively activated at only one site on the membrane to cause a localized deposition of chitin.

Methods Employed: Transmission electron microscopy is being used for morphological studies of fixed and embedded cells and for examination of purified proteins by negative staining. Phase and fluorescence light microscopy are used to monitor living cells. Standard biochemical procedures are used for enzyme activity and protein measurements. Cytochemical staining of thin sections was performed using colloidal gold stabilized with appropriate ligands. Monoclonal antibodies are being made by standard techniques.

Major Findings:

(1) In previous years we have achieved isolation of relatively pure fractions of Acanthamoeba plasma and vacuolar membranes. In the current year we have used these membrane fractions to immunize mice with the aim of producing monoclonal antibodies that are specific to each set of membranes. We are currently screening clones from the hybridomas to determine whether we have succeeded in producing appropriate antibodies. The goal is to use specific monoclonal antibodies with EM cytochemical techniques for study of membrane recycling.

(2) Cabib and co-workers have isolated, characterized, and obtained in vitro synthesis of chitin by a synthetase from Saccharomyces. As part of a continuing study of chitin synthetase in this organism, a set of cells mutant for chitin synthetase have been examined in the electron microscope. M.L. Slater produced a mutant yeast in which chitin synthetase activity was not detectable by biochemical assay for activity (aut^-). A transformant of aut^- in which multiple copies of the putative chitin synthetase gene (aut^+) were inserted via a plasmid was obtained. This transformant showed greatly increased chitin synthetase activity in the biochemical assay. In a third variant the chitin synthetase gene of a wild type yeast was "knocked out" by insertion of an interrupted (non-translatable) aut^- gene. This latter mutant (produced by P. Robbins) also lacked biochemically detectable synthetase activity but contained a normal amount of chitin. All three mutants grow at an approximately normal rate and show no gross abnormalities at the light microscope level, but have decreased viability. In order to better understand the cellular effects of the loss of synthetase activity, we examined the mutants for fine-structural morphology and used a cytochemical stain for chitin to look for the presence and distribution of chitin in the cell wall. We made 10 nm colloidal gold and coupled it to wheat germ agglutinin for a chitin specific cytochemical probe. The three mutants proved to be very similar in appearance at the EM level. All contained some morphologically normal cells but aut^- and the transformant contained 40-60% cells with abnormally thickened cell walls. In the thick-walled cells chitin

was found through out the wall rather than confined to the septal areas as is normally the case. The "knockedout" mutant displayed the greatest wall abnormality. Cells were misshapen, more frequently lysed and over 90% of the cells contained an abnormal distribution of chitin in the wall, although they were not greatly thickened. Neither the biochemical nor morphological results cannot be fully rationalized at present, but may indicate that there is an additional, heretofore undiscovered, chitin synthetase in the cell.

(3) In collaboration with other members of the Laboratory of Cell Biology (J.P. Albanesi, M. Coue, and H. Fujisaki) we have examined the interaction of myosin I with actin.

(a) Short actin filaments were produced by interaction with gelsolin (50:1 molar ratio) and mixed with myosin I in a 10:1 (actin:myosin I) molar ratio and the sample examined by negative staining in 1% uranyl acetate. The actin filaments complexed with gelsolin gave a range of lengths, from about 30-600 nm. The actin/gelsolin filaments generally appeared smooth and relatively straight. When myosin I was added in the absence of ATP, bundles of actin formed. The bundles were composed of numerous actin filaments and were generally fusiform in shape with tapered ends. The bundles were slender (0.1 - 0.6 μM diameter) and variable in length, but ranged to at least 6 μM, approximately 10 fold longer than the longest actin/gelsolin filaments observed. The bundles thus appear to be formed from straight side-to-side alignment of linearly overlapping actin filaments cross-linked by myosin I. Myosin, not as well resolved as actin monomers in the filaments, presumably is represented by small lumps along the actin filaments. In the absence of myosin, actin filaments often lie in close contact along their lengths. Center to center spacing was measured in one instance at 78 A, which is then equal to the width of a single filament. In the bundles the actin filaments appear to be separated by a regular distance, measured in one instance at 147 A. Thus, the space between actin filaments measures 69 A or about the dimension of one myosin I molecule as determined by hydrodynamic measurements. In favorable images the periodicity of the crosslink along the filament appears to be slightly longer, on the order of 25 nm.

(b) Actin, mixed with myosin and allowed to condense within a capillary tube to form a thread, was fixed, embedded and thin-sectioned to ascertain the morphology of the contracted thread. Two fixation methods that have been reported to enhance visibility of actin in cells were compared. Segments of the fixed threads were embedded so that cross and longitudinal sections could be studied. Longitudinal sections showed short lengths of longitudinally aligned actin filaments occur, similar in appearance to the bundles seen in short actin/myosin mixtures by negative staining. There were cross-sections of single actin filaments and small bundles within the same image indicating that many filaments are oriented at right angles to the bundles. Stereo pairs of thread cross-sections show the thread to be an anastomosing network of long actin filaments that appear to align longitudinally to form small bundles randomly within the network.

Publications:

Zlotnik, H., Fernandez, M.P., Bowers, B. and Cabib, E.: Saccharomyces cerevisiae mannoproteins form an external year of cell that determines wall that determines wall porosity. J. Bacteriol. 159: 1018-1026, 1984.

Cabib, E., Kang, M.S., Bowers, B., Elango, N., Mattia, E., Slater, M.L., and Au-Young, J.: Chitin synthesis in yeast, a vectorial process in the plasma membrane. In: Microbial Cell Wall Synthesis and Autolysis. C. Nombela, ed., Elsevier Science Publishers, B.V. 1984. pp. 91-100.

Slater, M.L., Bowers, B., and Cabib, E.: Formation of septum-like structures at locations remote from the budding site in cytokinesis-defective mutants of Saccharomyces cerevisiae. J. Bacteriol. 162: 763-767, 1985.

Kuznicki, J., Cote, G.P., Bowers, B., and Korn, E.D.: Filament formation and actin-activated ATPase activity are abolished by proteolytic removal of a small peptide from the tip of the tail of the heavy chain of Acanthamoeba myosin II. J. Biol. Chem. 260: 1967-1972, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL- 00501-12 LCB

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October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Actin Polymerization

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	Altaf A. Lal	Guest Worker	LCB, NHLBI
	Dominique Pantaloni	Expert	LCB, NHLBI
	Marie-France Carlier	Expert/Guest Worker	LCB, NHLBI
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.8	4.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous studies have shown that ADP-actin undergoes an equilibrium polymerization with a critical concentration of 8 μM (in 1 mM MgCl-2 and 0.2 mM ADP) while ATP-actin is a steady state polymer with a critical concentration of 0.35 μM (in 1 mM MgCl-2 and 0.2 mM ATP). The hydrolysis of ATP that accompanies polymerization of ATP-actin occurs on the filament subsequent to, and slower than, the polymerization step so that the actin filament in ATP has an ATP-cap at one or both ends. It is this ATP-cap that stabilizes the filament, which consists mostly of ADP-actin subunits. We have now found that an equilibrium, all ATP-actin polymer can be transiently formed when high concentrations of actin are polymerized under continuous sonication. This transient polymer has a critical concentration of 3 μM, suggesting that the steady state polymer is stabilized by the heterologous interaction of ATP-actin and ADP-actin subunits at the interface between a short ATP-cap and the ADP-core. This was confirmed by showing that the rate of nucleation and elongation of mixtures of ATP-actin and ADP-actin were faster than the rates with either species alone. These, and other, studies have led to a new model for actin polymeriztaion which accurately predicts the experimental polymerization curves. In addition, experiments were carried out with the barbed filament ends blocked with either gelsolin or cytochalasin. In this way, it was found that only the barbed end, and not the pointed end, has an ATP-cap at steady state while both ends may be capped at monomer concentrations greater than the critical concentration. These experiments also allowed calculation of the association and dissociation rate constants and the critical concentrations for both the barbed and pointed ends of the equilibrium ADP-actin polymer and the steady-state ATP-actin polymer.

Project Description

Objectives: Actin, is a monomeric protein of 42,000 daltons that polymerizes to long helical filaments. These filaments are the principal components of the membrane-cytoskeletal complex of all eukaryotic cells and together with myosin provide the mechanism for many motile activities. The state of polymerization of actin is under dynamic control especially in non-muscle cells. We are studying the mechanism of polymerization of actin, the role of ATP hydrolysis in the polymerization process and the regulation of polymerization by other proteins that interact with actin monomers, actin polymers or both.

Major Findings: In past years, we have found that the polymerization of actin can be divided into three phases: a slow nucleation phase leading to the formation of a thermodynamically unstable trimer, a rapid elongation phase in which monomers add to both ends of the trimer forming long filaments with little or no accumulation of oligomers of intermediate size, and a slow filament-length redistribution phase in which the length distribution becomes more narrow by the loss of monomers from some filaments and the addition of monomers to other filaments with no change in the concentration of total polymers. Most of our work this year has been concerned with the elongation phase about which considerable detailed information has now been acquired.

In the simplest model for reversible polymerization, the rate of filament elongation is:

$$dF/dt = k_+N - k_-Nc_t = k_+N(c_t - c_c) \quad (1)$$

where N is the concentration of elongating filaments, k_+ is the association rate constant, k_- is the dissociation rate constant, c_t is the monomer concentration at time t, and c_c is the concentration of monomer at equilibrium (k_-/k_+). Experimental data were obtained either from spontaneous polymerization (in which N must be estimated by computer fits using appropriate mathematical models) or from seeded polymerization (in which N is fixed by addition of a known concentration of preformed filaments). Polymerization was monitored by the increase in light scattering or fluorescence of suitably labeled actin. These methods have been useful in confirming the correctness of the general model and for determining rate constants. However, because N, the concentration of filaments, is only nanamolar while c, the concentration of monomers, is micromolar, these methods provided little information about the nature of any intermediates in the polymerization process. Specifically, little could be learned about the composition of the elongating filament end in ATP. Therefore, a new experimental method was developed this year.

Polymerization has been followed under continuous sonication. The assumption was made, and experimentally justified, that filaments are maintained at a constant length during sonication. Therefore, polymerization under sonication results in an increase in the concentration of filaments of constant length rather than an increase in the length of filaments of constant concentration, as occurs in the absence of sonciation. Under sonication:

$$N = c_w/\bar{m} \quad (2)$$

where c_w is the concentration of polymerized actin subunits and $\bar{m}$ is the

average filament length under sonication. With this value for N, equation (1) can be written:

$$dF/dt = (k_+/\bar{m})(c_0 - c_t)(c_t - c_c) \quad (3)$$

where c_0 is the total concentration of actin subunits. Equation (3) describes a symmetrical polymerization process which begins slowly, reaches a maximum rate at $t_{1/2}$ (the time when polymerization is half-completed), and then decreases to zero when the actin monomer concentration decreases to c_c.

The polymerization of ADP-actin is an equilibrium polymerization. It was demonstrated that the experimental data for polymerization of ADP-actin under continuous sonication (monitored by the increase in fluorescence of NBD-labeled actin) exactly fit the theoretical curve, i.e. the polymerization curve was symmetrical about the mid-point, identical (when normalized) for all concentrations of ADP-actin, and linear on a semi-log plot. Moreover, a plot of the maximum rate of polymerization (i.e. the rate at $t_{1/2}$) extrapolated to zero at 8.3 μM actin which gives a value for the c_c of ADP-actin identical to that obtained by more traditional methods.

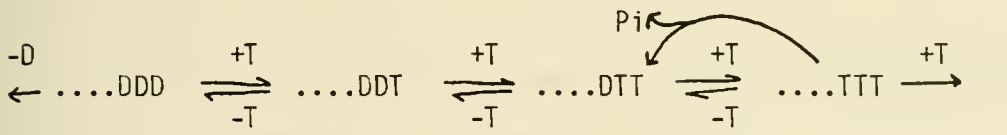
With this justification of the method, we then analyzed the polymerization of ATP-actin under continuous sonication. The polymerization of ATP-actin is a steady state, not an equilibrium process, because of the continual hydrolysis of ATP and equations (1) and (3) are not expected to apply. We showed previously that ATP is hydrolyzed on the filament subsequent to the polymerization step and that, because ATP hydrolysis is slower than monomer addition, there exists at steady state an ATP-cap at the end of the filament that consists otherwise entirely of ADP-subunits. The critical concentration for ATP-actin at steady state is 0.35 μM under the conditions of our experiments.

When ATP-actin at high concentration was polymerized under continuous sonication we obtained symmetrical polymerization curves to a transient plateau followed by a slow depolymerization, i.e. ATP-actin polymerization under these conditions was initially a pseudo-equilibrium process. This was because the rate of polymerization was so greatly accelerated by sonication that it was completed before significant ATP hydrolysis had occurred. Thus, an actin filament was transiently formed which contained essentially only ATP-subunits. Analysis of the data for this equilibrium polymerization of ATP-actin gave a c_c = 3 μM, much higher than the steady state c_c of 0.35 μM for ATP-actin.

The fact that the c_c for the ATP-actin equilbrium polymer was much higher than for the ATP-actin polymer at steady state suggested that the ATP-cap at steady state must be very short and that filaments with a short ATP-cap are more stable than filaments with a long stretch of ATP-subunits. This implies that the heterologous interaction between the ATP-actin and ADP-actin subunits at the interface between the ATP-cap and the ADP-body of the filament must be stronger than the homologous interactions between ATP-actin subunits only or between ADP-actin subunits only. This was tested by studying the copolymerization of ATP-actin and ADP-actin. It was found, in confirmation of the hypothesis, that the rate of nucleation and the rate of elongation were faster for copolymerization than for polymerization of ATP-actin or ADP-actin alone.

These data, and data reported in previous years, led us to a new hypothesis for

the detailed events of actin polymerization in ATP. The end of the growing filament can be considered to have several possible configurations, considering only the three terminal subunits because of all other subunits are in an environment approximating that of the third subunit from the filament end. We consider the following four confirmations to be most likely:....DDD, ...DDT, ...DTT, and ....TTT. The relative concentrations of the four species will depend on the concentration of monomeric ATP-actin. When only monomeric ADP-actin is present only ....DDD ends are possible and these have a c_c of 8 μM. At very high concentrations of ATP-actin only ....TTT ends (and longer stretches of ATP-subunits) are possible and these have a c_c of 3 μM. At lower concentrations of ATP-actin, ...DDT and ....DTT become the dominant species and the c_c is 0.35 μM. The polymerization process becomes:



(4)

Polymerization uncoupled from ATP hydrolysis would involve the addition of a T-subunit with lengthening of the ATP-cap. Polymerization coupled to hydrolysis would maintain the same length of ATP-cap but increase the length of the ADP-core. Depolymerization would be the loss of ADP-subunits (after initial loss of the short ATP-cap).

Detailed mathematical treatment of this model produced computer-generated elongation curves that precisely fit the experimental data. In particular, the model explains why polymerization is found experimentally to be faster than expected as the critical concentration is approached. At high actin concentration, elongation is faster that ATP hydrolysis, the filament has long stretches of ATP-subunits and the polymerization is proceeding towards the c_c of 3 μM characteristic of the ATP-equilibrium polymer. As the monomer concentration becomes lower, the rate of polymerization becomes slower and ATP hydrolysis on the filament reduces the size of the ATP-cap. Now, polymerization proceeds towards the steady state c_c of 0.35 μM and, therefore, introduces an asymmetry into the polymerization curve.

In the above discussion, we have treated the kinetics of actin polymerization as if there were only one elongating end. In fact, there are two and, therefore, equation (1) is more properly written as:

$$dF/dt = (k_+^b + k_+^p)\,N - (k_-^b + k_-^p)Nc_t = k_+^b N(c_t - c_c^b) + k_-^p N(c_t - c_c^p) \qquad (5)$$

where b and p refer to the barbed and pointed ends of the filaments, respectively. For the equilibrium polymer of ADP-actin (or ATP-actin), c_c will be the same at the two ends but for the steady state polymer c_c can be (and is) different at the two ends. For both equilibrium and steady state polymers, k_+ and k_- can be (and are) different at the two ends.

When polymerization kinetics are studied in bulk solution, the measured association and dissociation rate constants will be the sum of the values for the two ends and the measured critical concentration will be the sum of the dissociation

rate constants divided by the sum of the association rate constants. To determine the individual values for each end it is necessary to block one end specifically thus allowing the values for the other end to be determined and, by difference, the value for the other end to be calculated.

The rate constants and critical concentrations can be determined experimentally by measuring the rate of elongation or dissociation of a known concentration of actin filaments when added to different concentrations of G-actin. From equation (1), it can be seen that the slope of the plot is k_+N and the intercept at zero actin ($c_t = 0$) is $-k_-N$. The actin concentration at which neither elongation nor depolymerization occurs is the critical concentration ($c_t = c_c$).

We have used plasma gelsolin and the drug cytochalasin D to block the barbed ends of actin filaments and thus obtain kinetic values for the pointed ends alone to compare to the values for barbed plus pointed ends obtained in the absence of the barbed-end blockers. The same conclusions were reached with both sets of experiments. The kinetic constants determined in these experiments, combined with previous information, are summarized in the following table for the conditions 5 mM Tris, pH 7.8, 0.2 mM dithiothreitol, 0.1 mM $CaCl_2$, 0.2 mM ATP or ADP, 1 mM $MgCl_2$.

Actin Polymer	Pointed End			Barbed End		
	c_c	k_-	k_+	c_c	k_-	k_+
	μM	s^{-1}	$M^{-1}\ s^{-1}$	μM	s^{-1}	$M^{-1}\ s^{-1}$
ATP-actin (equilibrium)	8	0.4	5×10^4	8	6.0	7.5×10^5
ATP-actin (steady-state)	4	0.4	1×10^5	0.14	0.23	1.7×10^6

From the shape of the curves of elongation rate vs actin concentration when the barbed filament ends were blocked, it was deduced that, the pointed end is not capped at the critical concentration in the presence of ATP. This is supported by the fact that k_- for the pointed end in ATP is the same as k_- for the pointed end in ADP suggesting that ADP-actin is the dissociating species even in the presence of ATP. Of course, as previously shown, the barbed end has an ATP cap at the critical concentration. At high actin concentrations, i.e. above the critical concentration the pointed end as well as the barbed end probably has an ATP-cap. Thus, early in polymerization, scheme (4) applies at both ends of the filaments but near and at the critical concentration, when the system is approaching or is at steady state, the dominant species at the pointed end is ....DDD while, as stated before, it is ....DDT and ....DTT at the barbed end.

Proposed course of research: We will study in detail the interactions of several actin-binding proteins with actin in ATP and ADP. These will include: plasma gelsolin, which binds to the barbed ends of filaments and to actin monomers forming a 1:2 (gelsolin:actin) complex; tropomyosin which self-associates and binds along both sides of the actin filaments; profilin, which binds to actin

monomers to form a 1:1 complex; and β-actinin, which binds to the pointed ends of filaments. We plan to study the binary mixtures and then to make ternary mixtures of pairs of actin-binding proteins with actin to determine if the properties of the ternary mixtures are predictable from the properties of the binary mixtures or if additional interactions occur when more than one actin-binding protein is present at the same time.

Publications:

Carlier, M.-F., Pantaloni, D., and Korn, E.D. Evidence for an ATP cap at the ends of actin filaments and its regulation of the F-actin steady state. J. Biol. Chem. 259: 9983-9986, 1984

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00503-13 LCB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure, Assembly and Function of Microtubules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

MAP-2, the most conspicuous protein (MW 270,000) in mammalian brain that coassembles with tubulin, has previously been found subject to multisite phosphorylation at 2 subsets of sites. The A subset, in the microtubule-projection domain, are occupied in MAP-2 isolated after assembly cycles; these sites have been labeled by intraventricular injection of 32-Pi, but the phosphatase and kinase involved had not been identified. The 10 B subset, primarilly in the binding domain, are unoccupied in MAP-2 thus isolated; they can then be phosphorylated by cAMP kinase. It seemed not unlikely that they might be phosphorylated also in vivo, since a third of this brain kinase is complexed with MAP-2.

We now have 2 phosphatase which can release the A subset, an intestinal alkaline phosphatase and an enzyme purified from brain using MAP-2 as assay substrate. Using MAP-2 thus depleted in A subset, we have determined which kinases can add additional residues after such phosphatase treatment. In addition to cAMP kinase, Ca/calmodulin and Ca/Plipid kinases were chosen initially because they are abundant in brain. The first 2 added a maximum of 10 residues regardless of prior phosphatase treatment. With Ca/Plipid kinase the plateau of "maximum" phosphorylation varied with enzyme concentration, but at both 20 and 30 residues, additional phosphates were added corresponding exactly to the amount removed by phosphatase.

We find MAP-2 rapidly isolated from adult rat brain has 30 phosphates, i.e. the A subset and 20 more, presumably released by phosphatase during assembly cycles. Incubation with cAMP kinase will show whether the latter 20 include the B subset.

The project concerning tubulin modificiation by tyrosinolation was resumed, using Crithidia in place of HeLa. We have also made progress toward purifying the brain detyrosinolating carboxypeptidase to homogeneity.

Project Description:

Objectives: Our objectives are to ascertain the biolgical functions of microtubule modification by tyrosinolation and phosphorylation.

Methods Employed: Biochemical procedures as indicated under Major Findings.

Major Findings:

1. MAP-2 Phosphorylation. MAP-2 is the most conspicuous protein (MW 270,000) in mammalian brain that coassembles in constant proportion to tubulin, and promotes in vitro polymerization of pure tubulin. MAP-2 isolated from assemblycycle purified microtubule protein contains 10 mol phosphate/mol. Since there are warm incubations in the assembly cycles, these phosphates might be phosphatase-resistant, and this was reported last year to be the case for every known type of protein phosphatase. This "A subset" of MAP-2 phosphate can only be phospho-labeled by turnover in living brain, as the phosphatase and kinase involved have not been identified.

A third of cAMP-dependent kinase in rat brain is complexed to MAP-2 and remains bound through assembly cycles. When twice cycled microtubule protein is incubated with γ-[^{32}P]-ATP, an additional 10 mol (total 20) is added to MAP-2. This "B subset" is labile to a variety of phosphatases.

Last year we reported preliminary evidence that these subsets could be further distinguished in that B sites were in both microtubule-binding and projection domains, whereas A sites were confined to the latter.

1a. Subsets of MAP-2 phosphate (S. Tsuyama, G. Bramblett). The B subset have now been localized more precisely, 6 in the binding domain (39-46 kDa chymotryptic fragments) and 4 in the 240 kDa projection domain. The A subset were distributed 9.5 in the latter, and only 0.5 in the binding domain. The figures for the A subset depend on the assumption that all 10 turn over comparably in vivo. There is some evidence for this in that rate and extent of ^{32}P release by a phosphatase (see below) were found to be the same as for total phosphate release. Determination of the distribution of the A subset was made possible by improved conditions for in vivo turnover, using intraventricular injection of ^{32}Pi; phospho-labeling was found to be less extensive using brain slices, intracranial injection in newborn rats, or tissue cultured from embryonic brain.

By PAGE analysis protease digests of MAP-2 labeled in A or B subset were shown to yield different labeled fragments, serving to rule out the possibility that 20 partially occupied sites are labeled in vivo, and become fully occupied in vitro.

Binding domain chymotryptic fragments of MAP-2 are identified by their ability to associate with microtubules when incubated with tubulin. An incidental finding was that the fraction of microtubule that does not then disassemble in the cold contains a characteristic array of low-molecular weight MAP-2 fragments.

1b. Phosphatases for MAP-2 (C. Patterson, S. Tsuyama). Using MAP-2 labeled in B subset as assay substrate a phosphatase has been purified from brain to near homogeneity. PAGE analysis showed comparable amounts of 4 polypeptides, 3 of which corresponded in molecular weight to the subunits of smooth muscle phosphatase 1. Although it released lable from A subset relatively slowly, it could be used to release the bulk of these phosphatases, in contrast to the results reported last year for other protein phosphatases. It may be the enzyme responsible for A subset turnover in vivo.

An intestinal alkaline phosphatase, unique among commercially available enzymes in being devoid of protease activity, has also been found able to release 2/3 of A subset phosphate from native MAP-2, (and all of A and B phosphate in the presence of dilute SDS). This has enabled a search for the kinase responsible for adding the A subset phosphates.

1c. Kinases for MAP-2. (G. Bramblett, S. Tsuyama). The catalytic subunit of cAMP-dependent kinase was purified from skeletal muscle, Ca/calmodulin kinase II was purified from rat brain and Ca/phospholipid ("C") kinase from brain was obtained from Dr. K.-P. Huang. Sephacryl S-400 fractionation also resolved a second brain Ca/calmodulin kinase, still contaminated with phosphatase. The observed rates (nmol/min x mg) of phosphorylation of MAP-2, and of a standard assay substrate, were: cAMP-dependent kinase, MAP-2 6000, histone 3000; CAM kinase II, MAP-2 250, synapsin 4000; C kinase, MAP-2 20, histone 2000.

To determine which of these could recognize the sites occupied by the A subset, we treated an aliquot of MAP-2 with alkaline phosphatase, giving substrates with about either 3 or 9 mol/mol. After inactivating phosphatase, both were maximally phospholabeled with a large amount of kinase, i.e. enough to reach a plateau in less than 30 min. At 160 units/ml (standard substrates) CAM kinase II added 12.8 mol/mol to the control, and 11.9 to the phosphatase treated. At 40 units/ml, cAMP kinase added 10.3 to the control, and 11.9 to the treated. At 150 units/ml, C kinase added 30 and 35.5, respectively, an increment of 5.5 balancing the 5.7 mol/mol previously released by phosphatase. At 60 units/ml the values were 14.8 and 20.9, an increment of 6.1 balancing the 6.4 released by phosphatase. Thus, of the 4 kinases, only C kinase appears to recognize the A subset phosphatase sites. The low rate at which this enzyme phosphorylated MAP-2 must be balanced against the colocalization with substrate in brain and in dendritic processes.

The B subset sites are vacant when MAP-2 is isolated after assembly cycles, and are defined by their accessibility to cAMP kinase. A similar approach is currently being employed to determine whether these sites may be phosphorylated in vivo. MAP-2 rapidly isolated from rat brain without warm incubations has been found to contain not 10, but 30 mol phosphate. We will determine whether this MAP-2 has diminished ability to be phosphorylated by cAMP kinase.

2. Tubulin tyrosinolation (S. Chang, G. Bramblett). The α chain of tubulin is subject to a unique post-translational modification, the ATP-dependent addition of tyrosine through peptide linkage to C-terminal glutamate. Tubulin tyrosine ligase has been purified to homogeneity (5000 fold) from brain. Since tyrosine in this position is also coded, the first modification must be its removal. The responsible carboxypeptidase (CPT) has been purified 300 fold from brain. However, there has been uncertainty about the

specificity and nature of this enzyme, which can not be measured in extracts, where it has very low activity. It appears possible that we have now purified CPT to homogeneity in a single further step, using an fplc sulfonic acid ion exchange column.

Crithidia fasciculata, a kinetoplastid protozoan which we have established in culture, affords a unicellular organism in which tyrosinolation can be studied. It also harbors a third post-translational modification, acetylation of flagella α chain.

Proposed Course of Research:

1. MAP-2 Phosphorylation. Immediate projects, indicated in Major Findings (1c), include a protocol for determining whether the B subset sites located in the binding domain can be phosphorylated in vivo, as they are in vitro by cAMP kinase.

Because of the molecules large size, protease digest maps have not so far given clear answers to whether the various kinases recognizes different sites. We have prepared a double isotope program giving ^{35}S and ^{32}P dpm in mixtures, and may use labeled ATP and ATP(γ)S for this (and other) purposes.

Since the only kinase so far shown to recognize the A subset sites is membrane-associated, brain particulate fractions should be examined for phosphatase specific for these sites.

Further along, we may incubate brain slices with first or second messengers, in an approach like that of the Greengard laboratory. The phosphorylation state of the MAP-2 is subsequently determined by the change in extent to which it can be phospho-labeled by the appropriately regulated kinase.

Once it is established which of the multisite phosphorylations observed with various kinases do occur in vivo, it will be appropriate to turn to their effects on MAP-2 function. Subject to the caveat that "cytoskeleton" is largely a euphemism for a collection of proteins of unknown function.

2. Carboxypeptidase-tubulin. Purification of this important enzyme will enable us to characterize its structure, function and specificity, and to address specific questions such as the basis for its preference for oligomeric and polymeric forms of substrate. Antibodies may make it possible to test for its presence in other tissues and cells, and during developmental changes, as well as its subcellular localization. In the absence of specific inhibitors, microinjection of antibodies offers a possible approach to the function of tyrosinolation.

3. Crithidia. Difficulty in purifying tubulin from HeLa cells has blocked progress for several years in addressing questions about tyrosinolation that can only be answered with a unicellular system. Crithidia are closely related to Leishmania, which we showed some time ago to tyrosinolate tubulin, and Crithidia extracts contain very large amounts of tubulin. The initial questions relate to the nature of "non-substrate" tubulin, and anomalous pulse-chase results.

The cytoskeleton has several unusual features in these protozoa: a microtubule basket directly underlying the plasma membrane, a flagella paraxial rod of unknown composition and function (absent in some strains), tandem repeats of α and β genes, C-terminal tyrosine in β as well as α chain, and possibly a 58 kDa MAP in taxol-microtubules.

Publications:

1. A.S.N. Murthy, G.T. Bramblett and M. Flavin. "The sites at which Brain Microtubule-associated Protein 2 is Phosphorylated in vivo differ from those accessible to cAMP-dependent kinase in vitro." J. Biol. Chem. 260: 4364-4370 (1985).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01-HL-00506-10 LCB

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October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Acanthamoeba myosins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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6.4	6.4	0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Considerably more information has been acquired about the three myosin isoenzymes of Acanthamoeba. A 16-amino acid sequence has been identified which contains the ATP-binding site of myosin II: Glu-Ser-Gly-Ala-Gly-Lys-Thr-Gln-Asn-Thr-Me_2Lys-Lys-Val-Ile-Gln-Tyr. Radioactive nucleotide is photoaffinity crosslinked to the Gln at position 8 in this sequence. The sequence is highly homologous to a sequence in nematode and rabbit striated myosins that lies in the previously identified "ATP-binding" region of those myosins but differs from the two sequences that are photoaffinity labeled in rabbit myosin by two different analogues of ATP. A 68-kDa head fragment has been purified from tryptic digests of myosin II and shown to have complete Ca-ATPase activity but no actin-activated MgATPase activity. It did not bind detectably to F-actin. When covalently crosslinked to F-actin by EDC, the 68-kDa showed very high actin-activated ATPase activity; suggesting that the low activity of the non-crosslinked head was due to its poor binding. It is likely that phosphorylation regulates the activity of intact, native myosin II in the same way, i.e. by affecting its binding to F-actin. Kinetic studies of the ATPase activity of myosin IA and IB as a function of myosin concentration at fixed actin concentration have led to the hypothesis that myosin I heavy chain contains two actin-binding sites. This hypothesis was supported by evidence that single molecules of myosin I crosslink F-actin (as measured by the increase in low-shear viscosity) and, in the presence of ATP and when the myosin I is phosphorylated, superprecipitate F-actin. Thus, monomolecular, non-filamentous myosin I can support contractile activity which means that the heavy chain of 125-130 kDa contains all that is required for contractile activity and that myosin filaments are not needed.

HS 6040 (Rev. 1/84) GPO 904-917

Project Description

Objectives: Myosins are actin-activated ATPases that, through their interaction with actin filaments, are responsible for many of the motile activities of eukaryotic cells. Muscle contraction is the most obvious and best studied example but myosins of non-muscle cells are equally important although less well understood. The amoeba Acanthamoeba castellanii has been shown by previous work in this laboratory to contain three myosin isoenzymes, each regulated by heavy chain phosphorylation (a regulatory mechanism discovered in this laboratory). Our present efforts are concerned with understanding the details by which the state of phosphorylation of the myosins heavy chains regulates their actin-activated ATPase activities and the mechanisms by which the energy released by the hydrolysis of ATP is converted into movement.

Major Findings: Acanthamoeba myosin II: Previous work has shown that myosin II consists of two heavy chains of 185,000 daltons and two pairs of light chains of 17,500 and 17,000 daltons. The heavy chains are phosphorylated at three serine residues that lie within an 11-amino acid sequence that begins just 8 residues away from the carboxyl tail and is at least 90 nm away from the ATPase and actin-binding sites that are regulated. Only the dephosphorylated myosin II has actin-activated ATPase activity. Because only filamentous forms of myosin II are enzymatically active, because phosphorylated myosin II can inactivate unphosphorylated myosin II in copolymers and because myosin II from which a carboxyl-terminal peptide of 66 amino acids (which included the phosphorylation sites) has been cleaved cannot form bipolar filaments and has little actin-activated ATPase activity, we previously proposed that phosphorylation regulates myosin II by affecting filament conformation.

The effects of limited tryptic cleavage of myosin II were consistent with this idea. Trypsin can cleave myosin II at a single site forming a head fragment of 75,000 daltons and a tail fragment of 110,000 daltons which are separable only under denaturing conditions. But, despite the fact that the nicked molecule still forms bipolar filaments it has little or no actin-activated ATPase activity. This suggests that the nicked molecules form filaments that are unable to attain the the active conformation even when the heavy chains are unphosphorylated.

More extensive studies have now enabled us to find conditions where trypsin produces a 68,000-dalton head-fragment free of the rest of the heavy chain. Complete Ca^{2+}-ATPase activity is retained but almost no actin-activated Mg^{2+}-ATPase activity remains in the 68-kDa head. The 68-kDa head has been extensively purified and then covalently crosslinked to F-actin by the zero-length crosslinker EDC, in about 20% yield. The crosslinked F-actin-head complex was then purified away from uncrosslinked material and found to have very high actin-activated ATPase activity.

Parallel studies showed that binding of both the chymotryptic-fragment (the molecules with only 66 amino acids removed from the tail) and the 68-kDa tryptic head to F-actin were almost undetectable under enzyme assay conditions while the native molecule was completely bound. Thus, these data indicate that the 68-kDa head is fully competent catalytically (as shown by the activity of the covalently crosslinked molecule) but that it has much poorer affinity for actin than does the native molecule. By extrapolation, we propose that the phos-

phorylated form of myosin II forms filaments that have a much lower affintiy for F-actin than do filaments of unphosphorylated myosin II and that this is the way in which phosphorylation inhibits actin-activated ATPase activity of myosin II. Because phosphorylated and unphosphorylated myosin II are both filaments under assay conditions it is difficult to compare their affinities for F-actin.

In other studies, the ATPase site was photoaffinity labeled by UV irradiation of myosin II in the presence of [^{3}H]-UTP, which had previously been shown to bind specifically to the active site of the enzyme. Peptides containing the covalently bound [^{3}H]-nucleotide were produced by limit tryptic or chymotryptic digestion and purified by a combination of procedures using different HPLC columns. The amino acid compositions and amino acid seuqences of the peptides were determined (the latter by automated Edmond degradation) and compared to the known sequences of rabbit muscle myosin and nematode muscle myosin and the partial sequence data that have been obtained in this laboratory for Acanthamoeba myosin II (by sequencing the genomic DNA). The peptide containing the bound nucleotide was shown to have the sequence:

Glu-Ser-Gly-Ala-Gly-Lys-Thr-XXX-Asn-Thr-Me_2Lys-Lys-Val-Ile-Gln-Tyr

where XXX represents the residue to which the UTP was attached.

This sequence is identical to a sequence in the myosin II gene where XXX is a Gln residue. The sequence is highly homologous with sequences in rabbit and nematode striated myosins (differing at only 2 positions) that are thought to be in the region of the ATP-binding site; but (although in the same general region of the heavy chain) it is different from the two specific sequences that others have found to be photoaffinity labeled in rabbit skeletal muscle myosin by two different analogues of ATP. Because this site was labeled directly with a nucleotide substrate (i.e. "zero-length") we think it must be very close to the pyrimidine ring of the nucleotide during the catalytic cycle.

The amino acid sequence of the C-terminal 85 amino acids of myosin II have been determined at the DNA and protein level. The sequence predicts that the tail region will adopt a coiled-coil structure, except for the terminal 28 residues that contain the phosphorylation sites that regulate actomyosin II ATPase activity. We have synthesized 4 peptides in this region and raised polyclonal antibodies to them. Antibodies raised against the coiled-coil region block actomyosin ATPase activity but antibodies raised against the non-helical region have no effect at the same concentration. Similarly, synthetic peptides with the sequence of the terminal portion of the coiledcoil region inactivate enzymatic activity while peptides lacking this region do not. We propose that actin-activated ATPase activity of myosin II is very sensitive to the conformation of the filament and that the conformation is regulated through the interaction of the terminal regions of the coiled-coil structure. Phosphorylation of the non-helical region would then, according to this model, modulate enzymatic activity by affecting the adjacent coiled-coil structure.

Acanthamoeba myosin I: Myosins IA and IB from Acanthamoeba are structurally atypical myosins in that they contain only a single heavy chain and a single light chain and in that the heavy chains are much smaller (130 kDa and 125 kDa vs about 200 kDa for other myosins). Most importantly, myosins IA and IB are

incapable of forming bipolar filaments that are generally thought to be essential for contractile activity of actomyosin complexes.

When myosin I ATPase activity is measured as a function of actin concentration, there is an initial activation phase, followed by an inactivation phase as the actin concentration is increased, and then a re-activation phase at still higher actin concentrations. Last year, we showed that these complex kinetics were the result of an actin-dependent myosin cooperativity such that the apparent affinity of the myosin for actin was greater at high myosin:actin ratios than at low myosin:actin ratios; i.e. less actin is required to activate at high myosin:actin ratios than at low myosin:actin ratios. This year we have been able to propose a reason for this phenomenon which can form the basis of the mechanical functioning of myosin I in contractile events.

Experimental data support the idea that the myosin I heavy chain may possess two actin-binding sites: a high-affinity site that is ATP-insensitive and not directly involved in catalytic activity and a low-affinity, ATP-sensitive site that is the catalytic site. At high myosin:actin ratios, myosin molecules would be bound to the actin filament through their high-affinity sites sufficiently close together to interact cooperatively. Specifically, when one myosin molecule happened to bind a second actin filament through its low-affintiy site in addition to its high-affinity site, the low-affinity site of a close neighbor would be brought into close proximity with the actin filament and would bind. Thus, at high ratios of myosin:actin, there would be a cooperative increase in the apparent affinity for actin at the low-affinity site which would result in activation of the myosin ATPase activity. At lower ratios of myosin:actin, binding of one myosin molecule would have no effect on the binding of another and the low-affinity site would not be bound to actin. This then would explain the first activation phase and the subsequent inhibition phase as the actin concentration is raised at fixed myosin concentration. The final re-activation phase would occur when the actin concentration was high enough to bind to the low-affinity sites non-cooperatively.

The kinetic hypothesis predicts that myosin I should crosslink actin filaments; at high myosin:actin ratios multiple crosslinks would occur relatively close together while at low myosin:actin ratios the crosslinks would be few and far between. We had already shown that this occurred by the increase in low-shear viscosity when myosin I was added to F-actin and this year these studies were extended to correlate actin-activated ATPase activity with superprecipitation (i.e. "contraction") of actin-myosin I filaments. Crosslinking occurred in the absence of ATP more strongly than in its presence and equally well for unphosphorylated and phosphorylated myosin I. But superprecipitation occurred only in the presence of MgATP and when the myosin was phosphorlated which is the only form of myosin I that shows actin-activated ATPase activity.

These results are very important in understanding the mechanism of actomyosin-dependent motility and contractility. All other myosins that have been previously studied consist of two heavy chains of about 200-kDa and form bipolar filaments that are essential for contractility in the classical sliding filament model. Our studies prove that a single heavy chain of 125-130 kDa can contain all of the essential structural elements and that, providing the heavy chain has two actin-binding sites, myosin filaments are not required for actomyosin contraction to occur.

Until this year, no organism other than Acanthamoeba had been shown to have myosin I-like enzymes. We have now found that Dictyostelium discoideum contains, in addition to a conventional myosin, an enzyme very similar to Acanthamoeba myosin I. The Dictyostelium enzyme has a single heavy chain of 117,000 and a native molecular weight of 150,000. The Dictyostelium myosin I heavy chain reacts with antibodies raised against the Acanthamoeba myosin I heavy chain kinase. As for Acanthamoeba myosin I, phosphorylation of its heavy chain by Acanthamoeba myosin I heavy chain kinase greatly enhances the actin-activated ATPase activity of the Dictyostelium enzyme. It is likely that myosin-I-like enzymes exist in protista generally and the possibility that they exist in higher organisms must be seriously considered.

Proposed Course of Research:

Myosin II: Research will continue along several lines. We must complete the studies with the synthetic peptides and with the antibodies raised against them to define as completely as possible the role of the C-terminal portion of the heavy chains in the actin-activated ATPase activity and to correlate this with the role of this region of the heavy chains in filament formation. We are also attempting to identify the actin-binding site on the myosin II heavy chain and the myosin-binding site on the actin by crosslinking the 68-kDa head to the actin (as before), then proteolytically cleaving the crosslinked molecule. The product containing the crosslinked peptide fragments from the actin and myosin will be identified and the composition and, if necessary, the sequence of the peptides will be determined and compared to the sequences of actin and myosin II heavy chain. The actin sequence is known and the myosin II sequence is now being completed at the DNA level.

Myosin I: Efforts will continue to cleave the myosin I heavy chain into two fragments each containing one of the two proposed actin-binding sites. The properties of the two peptides (actin-binding, actin-activated ATPase activity, etc.) will be carefully studied. Small peptides containing the phosphorylation and ATPase sites will be isolated and their sequences compared to those of myosin II and other myosins.

Publications:

Kuznicki, J., Atkinson, M.A.L. and Korn, E.D. Effects of limited tryptic cleavage on the physical and enzymatic properties of myosin II from Acanthamoeba castellanii. J. Biol. Chem. 259: 9308-9313, 1984

Coté, G.P., Robinson, E.A., Appella, E., and Korn, E.D. Amino acid sequence of a segment of the Acanthamoeba myosin II heavy chain containing all three regulatory phosphorylation sites. J. Biol. Chem. 259: 12781-12787, 1984.

Albanesi, J.P., Fujisaki, H., and Korn, E.D. Localization of the active site and phosphorylation site of myosins IA and IB. J. Biol. Chem. 259: 14184-14189, 1984.

Kuznicki, J., Coté, G.P., Bowers, B., and Korn, E.D. Filament formation and actin-activated ATPase activity are abolished by proteolytic removal of a small peptide from the tip of the tail of the heavy chain of Acanthamoeba myosin II. J. Biol. Chem. 260: 1967-1972, 1985.

Coté, G.P., Albanesi, J.P., Ueno, T., Hammer III, J.A., and Korn, E.D. Purification from Dictyostelium discoideum of a low-molecular weight myosin that resembles myosin I from Acanthamoeba castellanii. J. Biol. Chem. 260: 4543-4546, 1985.

Albanesi, J.P., Fujisaki, H., and Korn, E.D. A kinetic model for the molecular basis of the contractile activity of Acanthamoeba myosins IA and IB. J. Biol. Chem. 260: in press, 1985.

Fujisaki, H., Albanesi, J.P., and Korn, E.D. Experimental evidence for the contractile activities of Acanthamoeba myosins IA and IB. J. Biol. Chem. 260: in press, 1985.

Albanesi, J.P., Fujisaki, H., Hammer III, J.A., Korn, E.D., Jones, R., and Sheetz, M.P. Monomeric Acanthamoeba myosins I support movement of beads along actin cables. J. Biol. Chem. 260: in press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00509-04 LCB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Lysosomes and Hydrolase Secretion in Acanthamoeba

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Thomas C. Hohman	Staff Fellow	LCB, NHLBI
	Blair Bowers	Research Biologist	LCB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Two lysosomal hydrolases from Acanthamoeba castellanii have been isolated from ameba growth medium and highly purified. N-acetyl-β-hexosaminidase was purified approximately 3500 fold over the growth medium. The activity applied to SDS polyacrylamide gels ran as a single band of 54,000 molecular weight. On sizing columns the activity eluted with an apparent molecular weight of 110,000, suggesting that the enzyme is a dimer.

β-glucosidase was purified 6000 fold over the growth medium. The apparent molecular weight from both sizing columns and polyacrylamide gel electrophoresis was 84,000. On PAGE one major and several minor bands were found. Preparative gel electrophoresis was performed and the 84,000 MW band eluted for injection into rabbits.

Polyclonal antibodies to both purified hydrolases have been obtained.

HS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives: Using Acanthamoeba as a model system, our long-range objectives are to understand the mechanisms involved in the exchange between internal membrane systems and the plasma membrane. The kinetics and physiology of lysosomal hydrolase secretion is one window on membrane exchange since the hydrolase clearly enter the cell's digestive compartment and are also released into the external medium. Thus it is likely that they serve as a "marker" for membrane movement. This year the major thrust has been to isolate and purify several of the lysosomal hydrolases and to prepare antibody against the purified enzymes.

Methods Employed: Fluorometric and colorometric assays were used for quantifying enzyme activity. Lysosomal hydrolases were purified with a combination of polyacrylamide gel electrophoresis and column chromatography. Other standard biochemical procedures were utilized as necessary for the experiments.

Major Findings:

(1) Purification of two hydrolases. Acanthamoeba secrete approximately 17% of their cellular content of N-acetyl-β-hexosaminidase activity and 5% of their β-glucosidase activity into the culture medium per hour. Growth medium recovered from amoeba cultures grown to a density of 2 million cells/ml is an excellent starting material for lysosomal hydrolase isolation.

Between 85 and 95% of the activities of both N-acetyl-β-hexosaminidase (β-hex) and β-glucosidase (β-glu) were recovered after ammonium sulfate precipitation of the growth medium and were purified 47 fold over the growth medium. After resuspension in and dialysis against phosphate buffer, the material was passed over an ion exchange column. One hundred percent of the β-hex activity was recovered in the flow-through from the DEAE-cellulose column and 95% of the β-glu activity was eluted from the DEAE-cellulose column with a linear NaCl gradient of 1 - 150 mM in 10 mM phosphate buffer, pH 8.0. With this column β-hex and β-glu were purified an additional 4 to 12 fold, respectively.

An affinity column for β-hex was prepared by coupling 2-acetamido-E-aminocaproyl-2-deoxy-β-D glucopyranoside (a generous gift from N. Sharon) to CNBr-activated Sepharose 4-B. The flow through from the DEAE-cellulose column was adjusted to pH 6.0 and applied to this affinity column. The column was then washed with 0.02% Triton X-100 in 25 mM phosphate buffer, pH 6.0, followed by a wash with the same buffer containing 150 mM NaCl. β-hex eluted from this column with 40 mM N-acetyl-β-D-glucoside in 25 mM phosphate buffer and was purified 2700 fold over the growth medium. Recovery of β-hex from this column was between 75 and 80% of the total activity applied.

The β-hex activity recovered from the affinity column was vacuum dialyzed against 100 mM phosphate buffer, pH 7.0 and applied to an S-300 sizing column. The activity eluted with an apparent molecular weight of 110,000 daltons and was purified 3500 fold over the growth medium. With SDS-polyacrylamide gel electrophoresis the activity recovered from the S-300 column appeared as a single band with an apparent molecular weight of 54,000. Approximately 0.5 mg of purified β-hex was recovered from 6-liters of growth medium.

The β-glu activity eluted from the DEAE-cellulose column was dialyzed against 10 mM phosphate buffer, pH 7.0, and applied to a 70 ml hydroxyapatite column equilibrated with the same buffer. Ninety-eight percent of the β-glu activity was eluted with a 10 - 150 mM phosphate gradient at pH 7.0, and was purified an additional 3 fold (1500 fold purification over the growth medium).

The β-glu activity recovered from the hydroxyapatite column was concentrated on an Amicon P-10 filter and dialyzed against 25 mM Tris-acetate buffer, pH 8.1. This material was then applied to a chromatofocusing column prepared with polybuffer 94 resin and eluted wtih a mixture of polybuffer 96 (3%) and polybuffer 74 (7%) adjusted to pH 5.0 with acetic acid. With this column β-glu eluted at pH 6.3 and was enriched 2 fold over the starting material (3000 fold purification over the growth medium). The peak tubes were concentrated by vacuum dialysis against 100 mM phosphate buffer, pH 7.0 and applied to an S-300 sizing column. The activity eluted with an apparent molecular weight of 84,000 daltons and was purified 2 fold over the starting material (6000 fold purification over the growth medium). When aliquots of the peak fractions recovered from the S-300 column were analyzed by SDS PAGE one major band with an apparent molecular weight of 84,000 daltons and several minor contaminating bands were identified. Only the density of the 84,000 dalton major band reflected the enzymatic activity of the fractions. This major band could be separated from the minor bands on preparative gels. The major band was then cut from the preparative gels and electro-eluted from the polyacrylamide. The eluted material did not retain enzymatic activity. Approximately 0.15 mg of this 84,000 dalton protein was recovered from 6-liters of growth medium.

(2) Antibody production. Antibodies were produced in rabbits by injection of the purified hydrolases. β-hex was used as recovered from the S-300 sizing column and β-glu as recovered by elution from a preparative gel. Antisera have been collected but not further characterized.

Significance to Biomedical Research:

Lysosomal dysfunction has been implicated in a large number of mammalian diseases including genetic disorders as well as parasitic infections. Understanding normal lysosomal functioning and cellular traffic between lysosomes and the cell exterior will provide insight into more effective ways of treating parasitic diseases and lysosomal dysfunctions.

Proposed Course of Research:

This project will not be continued due to the departure of Dr. Hohman. We plan to use the antibodies in cytochemical studies.

Publications:

T.C. Hohman and B. Bowers, 1985. Vacuolar pH is a factor regulating hydrolase secretion. Eur. J. Cell Biol. (accepted for publication).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00510-04 LCB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Conformational State of the Acto·S-1 Complex

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Lois E. Greene	Research Chemist	LCB, NHLBI
Others:	Roger Craig	Asst. Professor	Univ. of Mass.
	John Heuser	Professor	Wash. St. Univ.
	Robert King	Summer Worker	Duke University
	Evan Eisenberg	Section Head	LCB, NHLBI

COOPERATING UNITS *(if any)*

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Section on Cellular Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2/3	2/3	1/6

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have proposed that during the actomyosin ATPase cycle, the myosin cross-bridge alternates between two major conformations, which differ markedly in their strength of binding to actin and in their overall structure. In one conformation, which occurs in the absence of ATP, myosin binds very tightly to actin at a 45° angle. In the second conformation, which occurs only transiently when ATP or ADP·Pi is bound to myosin, myosin binds weakly to actin at an angle postulated to be 90°. We have now obtained structural evidence for the two different conformational states by using cross-linked actin·S-1. Both by negative staining and by freeze etching techniques, electron micrographs of cross-linked actin·S-1 shows that the overall structure of the cross-linked actin·S-1 is very disordered in the presence of ATP, with individual cross-linked S-1 molecules attaching to actin at variable angles centering on 90°. In the absence of ATP, the cross-linked actin·S-1 shows the typical arrowhead appearance, characteristic of the 45° conformation. The structure of the cross-linked complex was also examined in the presence of the ATP analog, AMP-PNP. In contrast to ATP, the structure of cross-linked actin·S-1 appears very rigor-like in the presence of AMP-PNP suggesting that the structural change induced by ATP is specific. In addition to these structural studies, we have also obtained biochemical evidence that cross-linked actin·S-1 can exist in two different conformations by studying the effect of troponin-tropomyosin on the cross-linked filament. We found that the conformation of cross-linked actin·S-1 depends on the nucleotide bound to the S-1. On the other hand, cross-linked actin·pPDM·S-1 and cross-linked actin·NEM·S-1 always remain in the 90° weak-binding and 45° strong-binding conformations, respectively, regardless of the nucleotide bound to S-1. This is in agreement with our previous studies which show that, irrespective of the nucleotide bound to S-1, pPDM·S-1 and NEM·S-1 form with actin stable analogs of the 90° and 45° conformations, respectively.

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Project Description

Objectives: In our model of muscle contraction, as ATP is hydrolyzed the attached myosin cross-bridge oscillates between the 45° strong-binding conformation and the 90° weak binding-conformation. There has been little information, however, as to the structure of the latter conformation, which occurs only transiently in the cross-bridge cycle when ATP or ADP·Pi is bound to myosin. By using S-1 which is cross-linked to actin we plan to study by electron microscopy, using negative staining or deep-freeze etching, the structure of cross-linked actin·S-1 in the presence of ATP. Cross-linked actin·S-1 has the key property of hydrolyzing ATP at a rate comparable to the maximal actin-activated ATPase of S-1, indicating that the cross-linked S-1 may undergo the conformational changes associated with the hydrolysis of ATP. Biochemical studies will also be conducted to study the conformations of different cross-linked actin·S-1 complexes. This will be done using the troponin-tropomyosin complex as a probe for the 45° and 90° conformations since this regulatory complex affects the two S-1 conformations very differently. Based on our model, we predict that when S-1 is cross-linked to actin it can oscillate between the 90° and 45° conformations, depending on the nucleotide bound to S-1. We also predict, based on our previous studies with pPDM-modified S-1 and NEM-modified S-1, that when these proteins are cross-linked to actin, the cross-linked complexes should only be able to exist in one of the two conformational states. We expect that, regardless of the nucleotide bound, the cross-linked actin·pPDM·S-1 and the cross-linked actin·NEM·S-1 should remain always in the 90° and 45° conformation, respectively.

Methods Employed and Major Findings:

Structural Studies:

The structure of the acto-S-1 complex in the presence of ATP was examined by electron microscopy. This was accomplished using both negative staining and a rapid freeze/freeze-etch technique to study a complex of S-1 covalently cross-linked to actin by the zero-length crosslinker, 1-ethyl-3-[3-(dimethylamino) proply]-carbodiimide. Two levels of S-1 binding were studied, with a molar ratio of cross-linked S-1 to total actin of either 20% or 50%. The lower percentage was used to observe individual S-1 molecules attached to actin, while the higher percentage was used to look at the overall pattern of S-1 decoration of the actin filament. In the absence of ATP, the appearance of both the 20% and 50% cross-linked filaments closely resembled the rigor appearance obtained using non-cross-linked proteins. The arrowheads observed had the conventional structure, and individual S-1 molecules were elongated, curved and appeared to make an angle of 45° with the thin filament. Both negative staining and freeze-etching showed that the addition of ATP to the cross-linked actin·S-1 complex caused a radical change in the structure of the cross-bridges. At both 20 mM and 170 mM ionic strengths, individual S-1 molecules appeared to be attached at variable angles which, in contrast to rigor, did not center on 45°. In addition, the S-1 molecules often appeared shorter and fatter than in rigor. The 50% cross-linked actin·S-1 preparation no longer showed the arrowhead pattern of S-1 decoration, but instead appeared disordered with little obvious polarity. Control experiments with ADP suggest that these effects were not due simply to a weakening of the binding of S-1 to actin in the presence of nucleotide, but were most likely ATP-specific. The cross-linked actin·S-1 complex,

which hydrolyzes ATP at about the same rate as the maximal actin-activated ATPase of S-1 (V_{max}), is composed of a mixture of states A•M•ATP and A•M•ADP•P_i, with more than 50% of the cross-linked S-1 occurring in state A•M•ATP. It therefore appears that in agreement with our model, both states A•M•ATP and A•M•ADP•P_i have a very different conformation from the classic arrowhead conformation of the A•M state.

The structure of cross-linked actin S-1 has also been examined in the presence of the ATP analog, AMP-PNP. In contrast to ATP, the addition of AMP-PNP had little effect on the rigor appearance of cross-linked S-1 even though it greatly weakened the binding of S-1 to actin. These results suggest that AMP-PNP is a rather poor analog of ATP, which is consistent with the X-ray diffraction pattern obtained with muscle fibers in the presence of AMP-PNP. They also suggest that the structural change induced by ATP is a specific effect and is not simply a staining artifact caused by a weak interaction of the cross-linked S-1 with actin.

Biochemical Studies:

Our previous studies suggested that, pPDM•S-1 forms a stable analog of the weak-binding conformation when it binds to actin, while NEM•S-1 forms a stable analog of the strong-binding conformation. In contrast, unmodified S-1 can exist in either of these conformations, depending on the nucleotide at the active site of S-1. In the present study, we crosslinked unmodified S-1, pPDM•S-1, and NEM•S-1 to actin in order to determine whether the respective properties of these different acto•S-1 complexes are maintained after cross-linking. The troponin-tropomyosin complex was used to probe the conformations of these different cross-linked complexes since the regulatory complex has very different effects on the 45° and 90° conformations. The cooperativity in the binding of non-cross-linked S-1•ADP to the regulated cross-linked filament was measured, as well as the inhibition of the ATPase activity of non-cross-linked S-1 by the regulated cross-linked filament. This showed that cross-linked NEM•S-1 turns on both the binding and the ATPase activity; cross-linked pPDM•S-1 turns on neither the binding nor the ATPase activity; cross-linked unmodified S-1 turns on the binding, but not the ATPase activity. This indicates that both in the presence of ADP and ATP, the crosslinked NEM•S-1 remains in the strong-binding conformation, while the crosslinked pPDM•S-1 remains in the weak-binding conformation. In contrast, crosslinked unmodified S-1 is in the weak-binding conformation in the presence of ATP and the strong-binding conformation in the presence of ADP. In confirmation of these results, SDS gel electrophoresis of the different cross-linked complexes show that cross-linked pPDM•S-1 has a different protein band pattern than that of cross-linked unmodified S-1 or cross-linked NEM•S-1.

Proposed Research: We will continue to study the structure of the cross-linked actin•S-1 complex by electron microscopy to determine the effect of different ATP analogs on its structure. These ATP analogs include PP_i, AMP-PNP plus ethylene glycol, and vanadate plus ADP. We will also examine the structure of the crosslinked pPDM•S-1$^-$ actin complex and the cross-linked NEM•S-1$^-$ actin complex in the presence and absence of ATP. These structural studies will look for conformations intermediate between the 45° and 90° conformations. If sufficiently ordered structures are obtained, 3-dimensional reconstruction techniques will be used to determine the structure of the acto-S-1 complex.

In addition, HMM, the two-headed fragment of myosin, will be cross-linked to actin. Structural and biochemical studies will be conducted on the cross-linked actin·HMM complex to determine whether only one or both heads of HMM are cross-linked to actin.

Publications:

Greene, L.E.: Stoichiometry of actin·S-1 cross-linked complex. J. Biol. Chem. 259, 7363-7366, 1984.

Craig, R., Greene, L.E., Eisenberg, E.: Structure of the Actin-myosin Complex in the presence of ATP. Proc. Natl. Acad. Sci, USA, 82, 3247-3251, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00514-02 LCB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Structure and Sequence of Non-Muscle Myosin Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John A. Hammer, III	Senior Staff Fellow	LCB, NHLBI
	Goeh Jung	Visiting Fellow	LCB, NHLBI
	Bruce M. Paterson	Research Chemist	LCB, NHLBI
	Edward D. Korn	Chief	LCB, NHLBI

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Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to isolate the genes encoding the non-muscle myosins of Acanthamoeba and to use the genes as tools to investigate myosin structure/function relationships and the in vivo functions of these cytoplasmic myosins. This project is part of the general effort in the Lab of Cell Biology to understand the organization and function of the cytoskeleton, using as a model system the soil amoeba Acanthamoeba. Acanthamoeba expresses simultaneously at least three distinct myosin enzymes, myosin IA, myosin IB and myosin II. Using molecular cloning techniques, we have isolated and purified a myosin II heavy chain gene and a myosin IB heavy chain gene. This study will provide, for the first time, the complete amino acid sequence of a non-muscle myosin. While non-muscle and muscle myosins share many common features, non-muscle myosins do possess unique structural, enzymatic, and regulatory properties. The amoeba myosin sequence data will be of great value in furthering our understanding of the unique structural and functional aspects of the amoeba myosins, and hopefully provide insight into the properties of cytoplasmic myosins in general.

The significance of this work is that by using the tools of molecular biology we can approach the study of these myosins in novel ways which are not possible using the classical techniques of protein chemistry. For example, we can use the genes to (1) make single determinant antibodies to synthetic peptides as probes of myosin function, (2) assign functional sites in the 1° sequence in combination with the amino acid composition of chemically crosslinked peptides, (3) search for cytoplasmic myosin genes in higher Eukaryotes, (4) alter the intracellular levels of myosin in the living cell as a way to study their roles in cell physiology, and (5) study structure/function relationships via site-directed mutagenesis of the gene.

GPO 914-918

Objectives:

(i) Immediate: Sequence the isolated myosin II and myosin IB heavy chain genes in their entirety. Analyze the deduced amino acid sequence in terms of (1) homology with sarcomeric myosin heavy chains, (2) secondary and tertiary structure predictions, (3) explanation at the sequence level for the unique properties of the amoeba myosins (eg. unusual structural properties of myosin IB, unusually small bipolar filaments formed by myosin II, cross-linking of actin by myosin IB), (4) identification of unique regions of myosin IB (i.e. different from "typical" myosins) to use at the DNA level as heterologous probes to search for myosin I - like genes in higher Eukaryotes, (5) identification of functional sites in the primary sequence in combination with the amino acid composition of chemically crosslinked peptides and (6) identification of areas from which to synthesize synthetic peptides in order to generate single determinant antibodies as probes of myosin function. Analyze the basic organization of the genes i.e. intron/exon pattern, sequence of regulatory regions, evolutionary relatedness.

(ii) Near Future: (A) Begin to work out details of expressing the myosin IB heavy chain in E. coli. This will require (1) removal of introns via oligonucleotide assisted gapping, (2) fusion of the full length intronless myosin IB sequence to an optimal bacterial expression vector, (3) selection of optimal growth conditions for high level expression of myosin IB protein and purification of myosin IB protein from E. coli and (4) using the computer-modelled tertiary structure of myosin IB (based on crystal structure of rabbit myosin S-1), design experiments to analyze myosin structure/function relationships by site-directed mutagenesis. (B) Begin to develop a means to reintroduce genes into amoeba. This requires fusion of a gene encoding a selectable marker to an amoeba myosin promoter and working out the details of a DNA-mediated transfection protocol for amoeba. In our first attempt we will use the gene for neomycin resistance, since amoeba are very sensitive to the aminoglycoside, neomycin. Eventually we will isolate an inducible promoter (perhaps the amoeba metallothionein promoter, inducible by adding heavy metals to the medium), which will allow us to regulate the expression of reintroduced genes. By inserting genes in both the sense and antisense orientation we can hopefully raise and lower the intracellular levels of myosins in the living cell, and therefore investigate the different roles these myosins play in normal cell physiology. (C) Use the different myosin II heavy chain genes (see below) to resolve the isoenzymes at the protein level (assuming that the multiple myosin II genes are all functional and give rise to significant amounts of protein-this will become clear as we analyze further the different myosin II heavy chain genes and look for evidence of heterogeneity at the protein level i.e. by 2-D mapping of purified myosin II proteolytic fragments). This approach will involve expression in E. coli of fusion peptides representing homologous portions of the various myosin II genes and use of these peptides to screen transient monoclonal antibody clones generated against purified myosin II for isoenzyme specific antibodies. These specific monoclonals can be used to fractionate the myosin II isoenzymes purified from amoeba as a mixture.

Methods Employed: Protein purification, antibody production and analysis by Western blot, purification of DNA and RNA, in vitro translation, immunoprecipitation, analysis of DNA fragments and RNA by restriction mapping and nucleic acid hybridization (Southern and Northern blots), cloning in plasmid

and bacteriophage vectors, construction of genomic libraries, hybrid selection analysis of cloned DNA, preparation and use of synthetic oligonucleotides, peptide synthesis, DNA sequencing.

Major Findings:

(A) We previously reported the isolation of a myosin II heavy chain gene from Acanthamoeba. This gene was identified by hybrid selection analysis, immunoprecipitation of hybrid-selected protein, Northern blot analysis, and DNA sequencing. During this reporting year we have:

(i) identified the 3' end of the gene (we identified the region of the gene that encodes the carboxy-terminus of the myosin heavy chain by using a mixed 20 bp oligonucleotide probe synthesized using a portion of the amino acid sequence of a carboxy terminal myosin II peptide (Cote et al., J. Biol. Chem. 259, 12751, 1984) - the amino acid sequence deduced from the nucleotide sequence of this region of the gene matched exactly the amino acid sequence of the C-terminal peptide, which had been sequenced by protein chemical methods - this result confirmed the identity of the clone, identified the 3' end of the gene, and provided transcriptional orientation),

(ii) approximately identified the 5' end of the gene by a combination of DNA sequencing, Northern blot analysis, primer extension analysis, and sequencing of primer extended products (we have yet to identify exactly the 5' end, but these results plus the DNA sequencing described in (iii) indicate that the myosin II genomic clone contains at least 90% of the coding information for the heavy chain and that the gene contains very little intron DNA),

(iii) sequenced about 25% of the gene (most of the sequence obtained is for the myosin head and shows $\sim$ 60% exact homology with other sequenced myosin heavy chains - this high degree of homology suggests that we could model the tertiary structure of the myosin II globular head using the crystal structure of rabbit myosin S-1), and

(iv) found evidence for 1 or perhaps 2 additional myosin II heavy chain genes in the amoeba genome (we probed digests of amoeba genomic DNA with the tail portion of the myosin II gene and saw evidence for additional highly related sequences in the amoeba genome - if there are additional myosin II genes, it is quite interesting because we have always thought of the purified myosin II protein as being a single polypeptide species - we have tentatively identified a second myosin II genomic clone by hybrid selection analysis, Northern blot analysis, and DNA sequencing).

(B) We previously reported the isolation of a myosin IB heavy chain gene. This gene was tentatively identified by hybrid selection analysis and Northern blotting. During this reporting period we have:

(i) confirmed the identity of the clone by specific immunoprecipitation of hybrid-selected protein and by DNA sequencing,

(ii) shown that despite the unusual structural properties of the myosin IB protein, the amino acid sequence so far deduced from the nucleic acid sequence shows that myosin IB is clearly related to typical sarcomeric myosins (we have

sequenced ~ 20% of the gene within regions identified using heterologous probes from the myosin II and nematode unc 54 myosin genes as containing functional areas - the deduced amino acid sequences encompassing the putative ATP binding site and active thiol in the myosin IB protein (totally ~ 250 amino acids) show 60% homology with typical sarcomeric myosin heavy chains)

(iii) shown by Northern blotting and DNA sequencing that the myosin IB heavy chain clone contains ~ 80% of the total coding information and that the gene appears to contain very little intron DNA, and

(iv) purified and are currently analyzing overlapping genomic phage clones which should contain the remainder of the gene.

Significance to Biomedical Research:

Using molecular cloning techniques, we have isolated and purified a myosin II heavy chain gene and a myosin IB heavy chain gene. This study will provide, for the first time, the complete amino acid sequence of a non-muscle myosin. While non-muscle and muscle myosins share many common features, non-muscle myosin do possess unique structural, enzymatic and regulatory properties. The amoeba myosin sequence data will be of great value in furthering our understanding of the unique structural and functional aspects of the amoeba myosins, and hopefully provide insight into the properties of cytoplasmic myosins in general. The significance of this work is that by using the tools of molecular biology we can approach the study of these myosins in novel ways including the analysis of structure/function relationships at the protein level by mutagenesis of the gene.

Proposed Course of Research: Continue to pursue the objective outlined above.

Publications:

Hammer, J.A. III, Korn, E.D. and Paterson, B.M.: Acanthamoeba Myosin IA, IB and II Heavy Chains are Synthesized In Vitro from Acanthamoeba Messenger RNA. (1984) J. Biol. Chem. 259: 11157-11159

Coté, G.P., Albanesi, J.P., Ueno, T., Hammer, J.A. III, and Korn, E.D.: Purification from Dictyostelium discoideum of a low-molecular-weight myosin that resembles myosin I from Acanthamoeba castellanii. (1984) J. Biol. Chem. 260: 4543-4546

Hammer, J.A. III, Korn, E.D., and Paterson, B.M.: Non-muscle myosin genes from Acanthamoeba. (1985) in Molecular Biology of Muscle Development, eds. Emerson, G., Fischman, D., Nadal-Ginard, B., and Siddiqui, M.A.Q., Alan Liss Inc. N.Y. (in press).

Albanesi, J.P., Fujisaki, H., Hammer, J.A. III, Korn, E.D., Jones, R., and Sheetz, M.P.: Monomeric Acanthamoeba myosins I support movement of beads along actin cables. (1985) J. Biol. Chem. 260 (in press).

ANNUAL REPORT OF THE
LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1984 to September 30, 1985

For some time, research in the Laboratory of Cellular Metabolism has been largely concentrated on the enzymes responsible for the synthesis and degradation of cAMP and cGMP through which many hormones, drugs, and other agents influence cellular function. During the two years work has been increasingly focussed on the hormone-sensitive adenylate cyclase. The objective of this effort is to elucidate the mechanisms for control of synthesis, assembly and operation of this ubiquitous regulatory system, which is basically analogous to certain other systems that serve in cell membranes to transduce stimuli from the environment through GTP-binding proteins to an internal effactor signal. In addition, work is continuing on specific cyclic nucleotide phosphodiesterases and calmodulin-regulated proteins as well as on the ADP-ribosyltransferases of animal cells.

1. Adenylate Cyclase: Role of GTP-binding Proteins in Signal Transduction

The hormone-sensitive adenylate cyclase system includes receptors for stimulatory and inhibitory ligands and stimulatory (G_s) and inhibitory (G_i) GTP-binding proteins through which agonist occupancy of receptors is translated into altered activity of the catalytic unit. G_s and G_i are heterotrimers consisting of a GTP-binding α-subunit ($G_{s\alpha} \sim 45$ kDa and $G_{i\alpha} \sim 41$ kDa) with identical β (35 kDa) and γ (8-10 kDa) subunits. The light sensitive phosphodiesterase of retinal rod outer segments resembles in many ways the cyclase system. Here, the photon receptor rhodopsin acts through transducin to increase cGMP phosphodiesterase activity. Transducin, like G_s and G_i, has a GTP-binding α-subunit ($T_\alpha \sim 39$ kDa) associated with β and γ subunits (35 and ~ 10 kDa); T_α and T_β are structurally similar to corresponding subunits of the G proteins. Because transducin is relatively easy to purify in reasonable quantities we have used it for several types of studies in the expectation that at least some of the results may be directly applicable to work on the cyclase proteins. With the goal of understanding at the molecular level the function of the cyclase system and the control of synthesis of its components, much of our effort is directed toward cloning the relevant genes. In addition,we are investigating structural, functional, and immunological relationships between G_s, G_i, transducin and two more recently recognized members of this family of GTP-binding regulatory proteins, G_o and H-ras p21.

From a bovine retina cDNA library provided by Dr. J. Nathans, we have isolated cDNAs that contain the entire coding region for T_γ. Northern blots of total RNA from bovine retina indicate one size class of T_γ message (~ 500 bases); no T_γ mRNA was detected in brain. Results of *in vitro* translation of total RNA from bovine retina and brain in a rabbit reticulocyte lysate system followed by immunoprecipitation with anti-transducin anti-serum were also consistent with the conclusion that T_γ is not expressed in brain.

Pertussis toxin catalyzes the ADP-ribosylation of $G_{i\alpha}$, $G_{o\alpha}$, and T_α, thereby altering their function. It had been reported that the site of modification in T_α was an asparagine located near the C terminus. The subsequently published cDNA sequence, however, indicated no asparagine in this position. To identify the modified amino acid, an ADP-ribosylated tryptic peptide was isolated from transducin radiolabeled with pertussis and [U-^{14}C-adenine]NAD. The sequence, glu-asn-leu-lys-asp-x-gly-leu-phe, corresponds to the cDNA sequence coding the C-terminal nonapeptide, which indicates cysteine in the "x" position. Cysteic acid was found on analysis of the performate oxidized peptide and a compound presumptively identified as ADP-ribosyl cysteine was recovered from the sequencing resin. Given the sequence homologies between T_α, $G_{i\alpha}$, and $G_{o\alpha}$, it is probable that this cysteine is the site of pertussis toxin-catalyzed ADP-ribosylation in all three proteins. Pertussis toxin is thus far the only known NAD:cysteine ADP-ribosyltransferase. We are attempting to identify in animal cells enzymes that catalyze an analogous reaction.

In cultured N1E-115 cells, muscarinic agonists (e.g., carbamylcholine) decrease cAMP content and increase cGMP. These cells contain a 41 kDa membrane protein that can be ADP-ribosylated by pertussis toxin, which is presumably $G_{i\alpha}$. Treatment of cells with pertussis toxin reduced the effect of carbamylcholine on cAMP levels without altering the cGMP response consistent with the conclusion that muscarinic effects on cGMP are not mediated through G_i.

Four monoclonal antibodies prepared against T_α, all react with a 23 kDa tryptic peptide from the N-terminal region. Three of these inhibited the GTPase activity of T_α reconstituted with $T_{\beta\gamma}$ and rhodopsin in phospholipid vesicles and did not cross-react with other GTP-binding proteins. The fourth monoclonal that did not inhibit GTPase activity but cross-reacted with $G_{i\alpha}$, $G_{o\alpha}$ and H-ras p21 may help in defining the relationship between the protooncogene product and these other GTP-binding proteins.

Interaction of T_α with the receptor rhodopsin requires $T_{\beta\gamma}$. It was unclear, however, whether both $T_{\beta\gamma}$ and T_α interact with rhodopsin or whether $T_{\beta\gamma}$ by interacting only with T_α increases its binding to rhodopsin. Since anti-idiotypic antibodies can mimic structure of the original antigen, we reasoned that some anti-idiotypic antibodies produced after immunization with transducin might resemble the site of transducin interaction with rhodopsin and would bind to rhodopsin. Immunization of rabbits with transducin or $T_{\beta\gamma}$ resulted in the appearance of anti-rhodopsin IgG that could be separated from anti-transducin or anti-$T_{\beta\gamma}$ IgG on transducin-Sepharose. Production of these presumably anti-idiotypic rhodopsin anti-bodies after immunization with $T_{\beta\gamma}$ provides evidence that $T_{\beta\gamma}$ interacts directly with rhodopsin. We showed last year that $G_{\beta\gamma}$ can replace $T_{\beta\gamma}$ in reconstituting rhodopsin-stimulated GTPase activity with T_α or $G_{i\alpha}$. Thus, it is likely that the site of $T_{\beta\gamma}$ binding to rhodopsin is very similar to the site of $G_{\beta\gamma}$ binding to inhibitory receptors. To investigate the latter interaction, we have begun to purify muscarinic receptors and have obtained a preparation that appears to be suitable for such studies.

Adenylate cyclase can be both activated and inhibited by F^- plus Al^{3+}, acting through G_s and G_i, respectively. Activation and inhibition by vanadate has also been reported. We, therefore, investigated the effects of F^- and vanadate on transducin function using purified T_α, $T_{\beta\gamma}$, and rhodopsin reconstituted in phosphatidylcholine vesicles. In this system, F^- inhibited GTP hydrolysis and inhibition was enhanced synergistically $AlCl_3$, which alone was only slightly inhibitory. Gpp(NH)p binding to T_α and release of bound GDP were also inhibited. Vanadate (decameric) similaryly inhibited GTP hydrolysis, GDP release, and GTP binding. ADP-ribosylation of T_α by pertussis toxin and binding of T_α to rhodopsin, both of which are enhanced by $T_{\beta\alpha}$, were inhibited by F^- with Al^{3+} and by vanadate. These findings are consistent with the conclusion that F^- plus Al^{3+} or vanadate can cause dissociation of T_α from $T_{\beta\gamma}$, resulting in inhibition of GDP-GTP exchange and thereby GTP hydrolysis. The dissociating effect of F^- (plus Al^{3+}) on transducin is analagous to its action on G_s and G_i which can result in adenylate cyclase activation or inhibition. Vanadate probably causes dissociation of G_s and G_i, as we have shown for transducin, accounting for its reported effects on cyclase activity.

2. Cyclic Nucleotide Phosphodiesterases

The so-called cGMP-stimulated phosphodiesterase hydrolyzes cAMP and cGMP with positively cooperative kinetics; at substrate concentrations below the apparent K_m, hydrolysis of one is stimulated by the other. Our earlier studies of the enzyme purified to homogeneity from calf liver showed that certain competitive inhibitors were also able to increase hydrolysis of low concentrations of substrate. After extensive analysis of kinetics of cAMP hydrolysis with and without cGMP or inhibitor, it appears that the data are best described by the rate equation for a two-site competitive allosteric enzyme model. In this model, the enzyme exists two states of "high" and "low" affinity; binding of substrate, effector, or certain inhibitors to the low affinity state induces allosteric transition to the high affinity state. Equilibrium binding constants for substrates and a number of methylxanthine inhibitors indicate that although several inhibitors bind as well as substrate to the low affinity state only two were nearly as effective as substrate in binding to the high affinity state, i.e., structural requirements are more stringent for binding to the high than the low affinity state. Temperature and pH alter allosteric transitions as well as catalytic activity. Low temperature (5°C) or high pH ($\sim$10) and $MgCl_2$ at 30°C promotes transition to the high affinity state in the absence of substrate, effector or inhibitor. All of our observations are consistent with the conclusion that this phosphodiesterase has independently regulated catalytic and allosteric sites with distinct topographical features.

Last year, we began investigation of the regulatory properties of the light-sensitive cGMP phosphodiesterase from retinal rod outer segments. Activity of the purified enzyme is increased > 10-fold by proteolysis with trypsin which inactivates the inhibitory 10 kDa subunit. We found that incubation with a purified ADP-ribosyltransferase from turkey erythrocytes plus NAD, which

modified both large and small units, increased activity albeit to a lesser extent than did trypsin. To define the mechanims of activation, we have compared the effects of ADP-ribosylation of the small vs. large subunits. There was no demonstrable effect of modification of the large subunits but it appears that ADP-ribosylation of the small subunit interferes with its ability to inhibit and thereby increases phosphodiesterase activity.

We reported last year that insulin activation of a particulate cAMP phosphodiesterase in 3T3-L1 adipocytes was prevented by prior treatment of cells with pertussis toxin, thus implicating a GTP-binding protein (G_i or G_o) in the process. Studies with relatively specific phosphodieterase inhibitors indicated that much of the anti-lipolytic effect of insulin may be secondary to the increased phosphodiesterase activity. Phenyl-isopropyl adenosine (PIA) a non-hydrolyzable analog of adenosine, was also anti-lipolytic and inhibited adenylate cyclase, as it does in some other cells. In addition, however, it activated the particulate cAMP phosphodiesterase and this effect, like that of insulin, was blocked by pertussis toxin. Both phosphodiesterase activation and adenylate cyclase inhibition presumably contribute to the antilipolytic effect of PIA. Experiments with adenosine deaminase were consistent with the conclusion that insulin activation of the phosphodiesterase is not mediated by adenosine.

3. Interaction of Calmodulin with Phosphodiesterase and other Proteins

Calcineurin, a calmodulin- and Ca^{2+}-binding protein, dephosphorylates phosphoseryl-, phosphothreonyl- and phosphotyrosyl proteins as well as p-nitrophenyl phosphate (PNPP). Last year we found that calcineurin activity was increased by dimethylsulfoxide (DMSO). Using PNPP as substrate, further studies revealed that in the presence of Mn^{2+}, 20% DMSO stimulated activity 200-400% compared with 100-200% stimulation by calmodulin. In the presence of Mg^{2+}, however, DMSO activation was only 50-100% and calmodulin-activated 8- to 12-fold. Effects of dimethyl formamide were very similar to those of DMSO, whereas formamide, ethanol, and methanol produced only small increases in Mn^{2+}-dependent activity. Thus, certain non-polar solvents, in the presence of Mn^{2+} but not Mg^{2+}, appear to permit expression of a "latent" phosphatase activity greater than that induced by calmodulin. Whether this reflects a mechanism for regulation of calcineurin activity by some intracellular factor(s) remains to be determined.

Calcineurin activity can be increased $\sim$ 100% by dithiothreitol (DTT), with a half-maximal effect at $\sim$ 1 mM. Treatment with DTT followed by its removal before assay did not increase activity; i.e., activation required the presence of DTT. By titration with dithionitrobenzene (DTNB), $\sim$ 4 reactive sulfhydryl groups were found in calcineurin. Addition of Ca^{2+} with or without calmodulin did not alter the extent of reaction with DTNB. Enzyme activity was unaffected by this modification. However, 2 µM $HgCl_2$ in the assay caused virtually complete inhibition. Inactivation by treatment with $HgCl_2$ before assay persisted after its removal but was reversed by DTT. Taken together, these observations suggest that although the sulfhydryl groups reactive with DTNB in the native enzyme are not critical for activity, there is a relatively inaccessible sulfhydryl that is important for catalysis.

Using dansyl-calmodulin, we had found that the interaction with phosphodiesterase occurred at concentrations of Ca^{2+} lower than those required for stimulation of enzyme activity. However, it is difficult to prepare sufficient phosphodiesterase to carry out a rigorous study of the two events and the necessary use of a poorly hydrolyzed substrate, which is fluorescent, complicates measurements of fluorescence polarization. After characterizing the activity of calcineurin vs. myosin light chain and myosin light chain kinase (phosphorylated on serine and threonine residues), the former was selected for use as a model substrate for analogous studies with the phosphatase. Since the activity of 0.5 μM calcineurin could be measured with PNPP or myosin light chains under conditions suitable for fluorescence measurements, calmodulin interaction and activation could be assayed in the same samples. With both substrates, the K_a for Ca^{2+} was 26-30 μM with apparent cooperativity for activation, whereas protein interaction required only 8-12 μM Ca^{2+} with no evidence of cooperativity. Thus it appears that calmodulin with Ca^{2+} bound at two high affinity sites can interact with calcineurin or phosphodiesterase but the complex is inactive until a third Ca^{2+} site is occupied. The affinity of the third site for Ca^{2+} may be markedly increased as a result of complex formation making enzyme activity particularly sensitive to changes in Ca^{2+} concentration.

Rabbit anti-phosphodiesterase IgG described last year and affinity-purified goat anti-calcineurin IgG were used in collaborative studies to map the distribution of these proteins in rat brain. Both were found only in nerve cells but their localizations, cellular and subcellular, were quite different. Calcineurin was found in both pre-synaptic and post-synaptic nerves in all regions. Staining appeared mostly along cell bodies, although axons and dendrites were also stained. In contrast, phosphodiesterase was highly concentrated in dendrites and somatic cytoplasm of large neurons that receive multiple convergent inputs (e.g., cerebellar Purkinje cells, pyramidal cells of cerbral cortex and hippocampus). Phosphodiesterase reactivity was homogeneously distributed whereas calcineurin immunoreactivity was observed in a striking punctate pattern along membranes of cell bodies and proximal dendrites. Such differences in the expression of calmodulin-dependent enzymes and their subcellular localization may well contribute to the specific functional properties of different neurons.

We have initiated investigation of possible changes in expression of calmodulin-binding proteins during phenotypic differentiation of pheochromocytoma (PC-12) cells and during the process of activation of different types of lymphocytes. A procedure for preparing biotinylated calmodulin (~ 2 mol/mol) and using it in conjunction with avidin coupled to peroxidase to detect calmodulin-binding proteins (e.g., on Western blots) was developed. This appears to have considerable advantages over the commonly used methods employing [^{125}I]calmodulin. With the biotinylated calmodulin and anti-sera against calcineurin and phosphodiesterase we have thus far characterized the calmodulin-binding proteins in undifferentiated PC-12 cells and in certain populations of resting lymphocytes.

4. ADP-Ribosyltransferases in Animal Cells

In the past few years, we have identified a family of NAD:arginine ADP-ribosyltransferases in animal cells. Although they catalyze the same model reactions as does choleragen, which in cells rather specifically modifies the α-subunit of G_s, their natural substrates are unknown. The mechanisms of the reactions catalyzed by choleragen and a highly purified transferase from turkey erythrocytes were investigated using agmatine as an ADP-ribose acceptor with and without the product inhibitor nicotinamide. Transferase and glycohydrolase reactions were monitored simultaneously using a mixture of [adenine-U-^{14}C] NAD and [carbonyl- ^{14}C]NAD as tracers. The data for choleragen were most consistent with a random rapid equilibrium mechanism whereas those for the erythrocyte transferase did not permit a distinction between this and a steady-state ordered mechanism.

If ADP-ribosylation plays a regulatory role in animal cells, it might be expected that there exist specific enzymes which remove the ADP-ribose moiety. To assay for such an enzyme activity, ADP-ribosylarginine was used as a model substrate. A cleavage enzyme activity has now been partially purified from turkey erythrocytes, in which we had earlier found several NAD:arginine ADP-ribosyltransferases. The products of the reaction, which is stimulated by Mg^{2+} and dithiothreitol, have been identified as ADP-ribose and arginine. With the presence of cleavage enzymes as well as transferases in cells, it appears that ADP-ribosylation may be a reversible modification of proteins involved in physiological regulation of function.

5. Regulation of cAMP and cGMP Metabolism in Intact Cells

Our earlier studies had shown that bradykinin acts through B-2 type receptors on cultured human fibroblasts to cause release of arachidonate and cyclooxygenase products that activate adenylate cyclase resulting in increased cell cAMP content. Bradykinin had little or no effect on fibroblast cGMP content. In cultured NG 108-15 (neuroblastoma x glioma hybrid) cells bradykinin caused a significant increase in cGMP; in N1E-115 (neuroblastoma) cells the effect was an order of magnitude greater. Analog potency was consistent with mediation through a B-2 type receptor. The rise in cGMP was not inhibited by indomethacin, a cyclooxygenase inhibitor, but was inhibited by ETYA, which inhibits both lipoxygenase and cycloocygenase. It appears that lipoxygenase metabolites may play a role in the bradykinin effect on cGMP.

6. Fatty Acid Metabolism in Adrenoleukodystrophy

We reported last year that cultured skin fibroblasts from patients with adrenoleukodystrophy (ALD) contin greater than normal amounts of very long chain fatty acids in cells from patients as well as normals. In subsequent collaborative studies, dietary supplementation with triolein significantly decreased serum hexacosanoic acid levels in two patients with ALD.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00606-14 CM

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of cAMP Content and Prostaglandin Production of Cultured Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Vincent C. Manganiello, M.D., Ph.D.	Head, Section on Biochemical Phydiology CM, NHLBI
Others:	Joel Moss, M.D., Ph.D.	Head, Section on Molecular Mechanisms CM, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous work has shown that bradykinin (BK) interacts with B-2 type receptors in human fibroblasts and initiates a series of biochemical events resulting in increased phospholipase activity, release of arachidonate and formation of prostaglandins predominantly via the cyclooxygenase pathway. These prostaglandins, in turn, activate adenyl cyclase and increase cAMP content. The effects of BK on fibroblast cAMP content can be inhibited by the cyclooxygenase inhibitor indomethacin.

BK also interacts with neuroblastoma cells and rapidly produces a marked and transient rise and fall in cAMP content. Analog potency suggests that the receptor is also of the B-2 type. In control to human fibroblasts, BK effects on cGMP content are not inhibited by indomethacin, but are inhibited by ETYA, an inhibition of lipoxygenase. These findings suggest that effects of BK on neuroblastoma cGMP content are perhaps mediated by lipoxygenase metabolites.

In human fibroblasts various factors alter responsiveness to BK. Incubation with pertussis toxin for 24 hrs enhanced BK-stimulated prostaglandin formation, as did other agents (including cholera toxin) that increased fibroblast cAMP content. The response to 8-Br cAMP was biphasic. During initial stages of incubation with 8-Br-cAMP, BK stimulation of prostaglandin formation was reduced. Longer incubations with 8-Br cAMP or 8-Br-cGMP (which did not reduce BK responsiveness) enhanced the effect of BK on prostaglandin formation. These findings support the hypothesis that cyclic nucleotides can modulate BK-stimulation of prostaglandin formation.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives: To elucidate mechanisms whereby hormones and other effectors influence cyclic nucleotide metabolism and prostaglandin production in cultured cells and vascular smooth muscle; to specifically study the mechanism of bradykinin (BK) effects on prostaglandin formation and subsequent stimulation of cAMP and cGMP accumulation.

Methods Employed: Cultured fibroblasts and neuroblastoma cells were grown and maintained under standard conditions. Cyclic nucleotides and prostaglandins were quantified by radioimmunoassay.

Major Findings: BK increased cGMP content of NG 108-15 and N1E-115 neuroblastoma cells. In the presence of BK, cGMP content increased rapidly, was maximal in ∿ 30-40 sec, and then declined. The response to maximally effective concentration of BK (1 nM) was 10-20 times greater in N1E-115 cells than in NG-108-15. Analog potency (lys-BK and BK > [Tyr^8]-BK; Des-Arg^9-BK not effective) suggests that the BK receptor on N1E-115 cells is of the B-2 type. The effect of BK on cGMP content was inhibited by ETYA (IC_{50} at ∿ 10 μM; almost complete inhibition at 10^{-4}M) but not indomethacin (ineffective at 10^{-4}M). Since ETYA inhibits both lipoxygenase and cyclooxygenase and indomethacin inhibits cyclooxygenase, these finding suggest a role for lipoxygenase metabolites in the effect of BK on neuroblastoma cGMP content.

In cultured human fibroblasts, BK interacts with a B-2 type receptor and initiates a series of events resulting in production of prostaglandins (via predominantly the cyclooxygenase pathway) which in turn activate adenyl cyclase and increase fibroblast cAMP content. A role for cAMP in the regulation of BK-responsiveness was postulated based on the observation that pertussis toxin, an agent that blocks activity of the inhibitory guanyl nucleotide-binding protein of adenylate cyclase, enhanced BK-stimulated PG formation. To characterize the action of cyclic nucleotides on BK-responsiveness, fibroblasts were incubated with cAMP and cGMP analogues prior to exposure to BK. PG were determined by radioimmuno assay. For the first several hrs of incubation with 0.5 mM 8-Br-cAMP, BK-induced PG formation was inhibited, following which the nucleotide stimulated PG formation by BK; at 0.02 mM 8-Br-cAMP, inhibition of BK-responsiveness was observed for up to 72 h incubation. 8-Br-cGMP did not inhibit when tested at concentrations of 0.0005 to 1.5 mM and for up to 72 hrs of incubation. Enhanced BK-responsiveness following a 6 and 24 h incubation with 8-Br-cGMP was greater than that observed with 8-Br-cAMP. By 72 h, the effect of 8-Br-cAMP on BK-responsiveness was greater than that of 8-Br-cGMP. These studies are consistent with the hypothesis that BK-stimulated PG formation is modulated by cyclic nucleotide content.

Significance to Biomedical Research and the Program of the Institute: Bradykinin, a potent stimulus for prostaglandin production in many cell types, is thought to play an important role in maintenance of vascular tone and permeability and in inflammatory processes. Human foreskin fibroblasts, the first human cells in which BK receptors have been characterized, and neuroblastoma

cells should prove useful for investigation of regulation of responsiveness to BK by receptor and post-receptor mechanisms as well as cellular mechanisms of action for vasoactive and hypotensive agents. Fibroblasts also constitute a good model system to study the coordinate and independent regulation of the responsiveness of the cyclase system to BK, hormones, and other effectors.

Proposed Course: Continued study on the mechanism of action of BK with emphasis on BK activation of the arachidonic acid cascade and regulation of cyclic nucleotide metabolism.

Publications:

Roscher, A.A., Manganiello, V.C., Jelsema, C.L., and Moss, J.: Autoregulation of bradykinin receptors and bradykinin-induced prostacyclin formation in human fibroblasts. J. Clin. Invest., 74: 552-558, 1984.

Jelsema, C.J., Moss, J., and Manganiello, V.C.: Effect of bradykinin on prostaglandin production by human skin fibroblasts in culture. In Birnbaumer, L. and O'Malley, B.W. (Eds.): Methods in Enzymology, Peptide Hormone Action. New York, Academic Press, 1985, Vol. 109, pp. 480-503.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00614-08 CM

PERIOD COVERED

October 1, 1984 through September 31, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Interaction of Calmodulin with Phosphodiesterase and other Binding Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Randall L. Kincaid, Ph.D.	Research Pharmacologist	CM, NHLBI
Others:	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI
	Sharon Geyer, Ph.D.	Guest Worker	CM, NHLBI
	Vsevolod Tkachuk, Ph.D.	Guest Worker	CM, NHLBI

COOPERATING UNITS *(if any)* Pennsylvania State University (M.L. Billingsley, C.D. Balaban); Laboratory of Immunology, NIAID, NIH (M.V. Sitkovsky). Dr. Tkachuk was a visiting scientist under the US-USSR Exchange in the Cardiovascular Area, Problem Area 3, Myocardial Metabolism.

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	1.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Ca2+-dependence of interaction of dansyl-calmodulin (CaM) with brain CaM-dependent phosphatase, calcineurin (CN), was compared with that of enzyme activation using identical experimental samples. Complex formation (fluorescence polarization) showed no cooperativity (K1/2 ∿ 5-6 μM Ca2+) while phosphatase activation was highly cooperative and required ∿ 3 times higher Ca2+ concentrations. Similar results were obtained for cyclic-nucleotide phosphodiesterase (PDE) using an alternative substrate, N6-etheno cyclic AMP. These results are consistent with a sequential mechanism of Ca2+-dependent interaction and enzyme stimulation wherein diffusion of Ca2+ is rate-limiting for activation-deactivation. Affinity-purified IgG fractions from rabbit (anti-PDE) and goat (anti-CN) showed < 1% cross-reactivity by ELISA procedures and did not react with other calmodulin-binding proteins (CaM-BPs), PDEs or other brain proteins using immunoblot analysis. The presence of CN and PDE in spleen cell populations and PC-12 cells was examined using these antibodies; in addition all CaM-BPs in these cells were visualized with biotinylated CaM (bioCaM), a new derivative developed for overlay procedures. The regional and subcellular distribution of PDE and CN in rat brain was investigated. Both CN and PDE were exclusively neuronal; glial and other non-neuronal tissue was unreactive. CN appeared to be ubiquitously distributed and was found in both pre- and post-synaptic structures; it exhibited distinct punctate immunoreactivity, especially along cell membranes (synapses). PDE was localized in dendrites and soma of a small subset of nerves which receive convergent input (cerebellar Purkinje cells, cortical and hippocampal pyramidal cells); it appeared entirely post-synaptic and was homogenously distributed in the cytoplasm. These data demonstrate cell-specific expression of CaM-BPs in the central nervous system and suggest a general role for CN in synaptic transmission while PDE may be involved in the integration of nervous input in selected neurons.

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Project Description:

Objectives: To determine the relationship between calmodulin (CaM) interaction with its binding proteins and enzyme activation, especially as regards the calcium dependence of these two events. To isolate and characterize the physical domains of interaction of CaM and its binding proteins. To prepare antibodies against these proteins for investigation of their *in vitro* and *in vivo* regulation.

Methods Employed: Homogeneous CaM, dansyl-CaM (D-CaM) phosphodiesterase (PDE), and calcineurin (CN) were prepared by previously described methods. Phosphorylated myosin light chains were a gift of Dr. James Sellers (Laboratory of Molecular Cardiology). Biotinylated CaM (bioCaM) was prepared by reaction of CaM with the caproic acid derivative of biotin in phosphate-buffered saline containing 0.1 mM Ca^{2+}. After 2 hrs at room temperature, the reaction mixture was dialyzed exhaustively against buffer to remove unreacted components. The UV spectrum of bioCaM showed a marked increase in absorbance at 260 nm and the degree of biotin incorporation was estimated from the increase in this absorbance.

The phosphatase activity of CN was measured either with the chromogenic substrate, p-nitrophenylphosphate (PNPP)(10-20 mM), as described previously or with the phosphoprotein substate, ^{32}P-myosin light chains (P-MLC). For most assays, CN was present at 0.1 - 0.5 μM and reactions (200 μl) were carried out in 20 mM Tris.HCl, pH 8.0, containing 0.2 mM $CaCl_2$ and 5 mM $MgCl_2$ in the presence or absence of 2 μM calmodulin. Release of phosphate from the light chains (~ 1 μM) was determined in the soluble fraction after termination of the assay with 20% TCA.

Antibodies were raised in rabbits (anti-PDE) and in goat (anti-CN) as described in a previous report. A purified anti-PDE IgG fraction was prepared from rabbit antisera by chromatography on Protein A-Sepharose. Briefly, crude antiserum was adsorbed to Protein A-Sepharose (1 ml serum/ml gel) and, after washing with 20 mM Tris-HCl, pH 8.0 containing 100 mM NaCl, the column was eluted with 2-3 bed volumes of 0.1 M Na acetate, pH 3.8 plus 10% glycerol and immediately neutralized with 2 M Tris-HCl, pH 9.0, to a final pH of 7.5. The concentration of IgG in this fraction was determined using an absorbance at 278 nm of 1.38 for a 1 mg/ml solution. The Protein A-Sepharose eluate was dialyzed against 10 volumes of 20 mM Tris.HCl, pH 8.0 containing 100 mM NaCl and 45% glycerol and stored at -20°. Affinity-purified antibodies against CN were prepared by adsorption of crude goat plasma on CN-Sepharose (2 mg purified CN coupled per ml of CnBr-activated Sepharose). After successive batchwise incubations of 0.3 - 0.4 liter portions of plasma with 10 ml of CN-Sepharose, the gel was sedimented and washed extensively with buffers containing 0.5 M NaCl, and 0.5% CHAPS prior to elution with the Protein A-Sepharose elution buffer. The IgG fraction of the affinity eluate was prepared by chromatography on Protein A-Sepharose, as described above, and stored at -20° after dialysis against buffer containing 45% glycerol.

The titer and specificity of antibodies were estimated by nitrocellulose blotting methods. After SDS gel electrophoresis of antigen or crude sample, proteins were transferred electrophoretically from the gel to nitrocellulose paper (Western blot). The paper was blocked with buffer containing 3% gelatin to reduce nonspecific binding, incubated with an appropriate dilution of antiserum or purified antibody (1:300-1:10,000) for 1-12 h, and then incubated with a detecting antibody specific for the species of the primary antibody covalently coupled to horseradish peroxidase; e.g., goat anti-rabbit peroxidase. Colored products appear after incubation with substrate.

Solid phase immunoassays were carried out in 96-well, polyvinyl microtiter plates. Antigen (10-200 ng diluted in 10 mM sodium bicarbonate, pH 9) was added to each well in a total volume of 30 µl and allowed to evaporate overnight. Wells were washed with phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA) in PBS. Dilutions of antibody were added to each well and incubated 1-16 hr at room temperature after which wells were washed with PBS. Detecting antibody (1:2000) was added, and after a 1 hr incubation, plates were washed and developed with appropriate chromogen. Immunoreactivity was quantified using an automated absorbance scanner.

Immunolocalization of CN and PDE was carried out on brain slices (50-100 µm) obtained from rats which had been perfused transcardially with 10% formalin in PBS. After washing of slices in PBS, overnight incubations (4°) were performed with primary antibody or pre-immune serum. The slices were washed with PBS containing 0.5% Triton X-100 and incubated with biotinylated second antibody for 1 hr at room temperature. The slices were incubated an additional hour with avidin-peroxidase and, after washing, developed with insoluble chromagen. Slices were mounted on slides, dehydrated through a graded series of solvent incubations, and photographed.

Rat pheochromocytoma (PC-12) cells were grown in 165 cm^2 culture flasks with DMEM media supplemented with 7.5% horse serum and 7.5 % fetal calf serum. After reaching confluency, cells were harvested and washed twice with 0.01 M potassium phosphate, pH 6.5 containing 0.25 M sucrose before freezing. Frozen cells were thawed in the presence of protease inhibitors and homogenized in 0.01 M Tris buffer, pH 8.0. The 20,000 xg supernatant was dialyzed against buffer containing 0.2 M NaCl and later chromatographed on QAE-Sephadex A-25 to remove endogenous CaM. After additon of Ca^{2+}, the QAE-treated supernatant was chromatographed on CaM-Sepharose. The fraction eluted with EGTA was precipitated with trichloroacetic acid for analysis by SDS gel electrophoresis and immunoblot procedures. Washed spleen cells or purified B, T or thymocyte fractions from mice were homogenized in buffer containing 0.25 M sucrose. The supernatant fraction from these cells was treated, as described above for PC-12 cells, to remove CaM and then to obtain the fraction eluted from CaM-Sepharose.

Major Findings: 1) Regulation of Phosphatase Activity of CN and the Mechanism of CaM-dependent Stimulation. In an extension of earlier studies regarding dimethylsulfoxide (DMSO) activation, several non-polar solvents were examined for their effects on the Mn^{2+}- and Mg^{2+}-dependent p-nitropheno phosphatase activity of CN. As seen previously, 20% DMSO stimulated the

Mn^{2+}-dependent activity ∿ 3-5 fold compared with ∿ 2-3 fold stimulation by CaM; by contrast, Mg^{2+}-dependent activity was stimulated only 1.5-2 fold by DMSO while CaM activation was 8-12 fold. Total CaM-stimulated activity in the presence of 0.5 mM Mn^{2+} was approximately twice that with 5 mM Mg^{2+}. Of the solvents tested, tetrahydrofuran, acetonitrile, tetramethyl urea, and methanol stimulated Mn^{2+}-dependent activity less than 40% while formamide and ethanol stimulated activity 60-80%. Dimethyl formamide (DMF) was a slighter better activator of Mn^{2+}-dependent activity than DMSO when directly compared; as with DMSO stimulation, maximal activity by DMF in the presence of Mn^{2+} was twice that seen with Ca^{2+}/CaM while for Mg^{2+} supported activity, DMF produced only 20% that observed with CaM. These data indicate that certain solvents can produce phosphatase activation greater than that seen with CaM, suggesting expression of latent enzyme activity; however, this high activity is only seen with Mn^{2+}, and not Mg^{2+}.

In collaboration with Dr. Vsevelod Tkachuk, studies on the role of sulfhydryl groups in phosphatase activity were initiated. As noted by others, reducing agents such as dithiothreitol (DTT) increased enzyme activity approximately two-fold, with a somewhat greater effect on CaM-stimulated than on "basal" (Mg^{2+}-dependent) rate. The half-maximal effect was observed at ∿ 1 mM DTT. Reduction of sulfhydryl groups followed by desalting (to remove DTT) did not increase activity suggesting that the enzyme required reductant during assay and that the lower activity did not reflect an oxidized form of the enzyme. Direct titration of sulfhydryl groups with dithionitrobenzoic acid (DTNB) indicated that there are ∿ 4 titratable groups available in the non-denatured protein; the addition of Ca^{2+} in the presence or absence of CaM did not appear to change the amount of DTNB incorporation. The activity of the DTNB-modified enzyme was essentially the same as that of the native enzyme. When 1-3 μM $HgCl_2$ was added to 0.5 μM CN in the assay, enzyme activity was virtually absent; this effect was reversed by 10 mM DTT. In addition, after treatment of CN with 10 μM Hg followed by desalting in buffers containing metal chelator, enzyme was completely inactive until DTT was added to regenerate activity, suggesting mercaptide formation. Taken together, these data indicate that sulfhydryl groups available in the native protein are not critical for enzyme activity, although the presence of reductant significantly increases reaction rate. However, since Hg can inactivate CN in a manner reversed by DTT, this may suggest a relatively inaccessible sulfhydryl group which is important for catalysis.

Previous studies with a mono-phosphotyrosyl form of glutamine synthetase (GS) showed a low rate of protein phosphatase activity (V_{max} = 10 nmol/min/mg), albeit with an affinity of 3 μM. Two other phosphoprotein substrates (myosin light chain (MLC) and myosin light chain kinase (MLCK)), which are phosphorylated at serine and threonine residues, were examined. Phospho - MLCK (2 mol P_i per mol protein) was dephosphorylated by CN with a velocity similar to that seen for GS. Although at equimolar concentrations of CaM and CN, velocity was maximal (stimulation, 6-8 fold), enzyme activity was reduced > 80% when CaM was present at five times higher concentrations, suggesting a direct effect of CaM on the availability of a phosphorylated site on the substrate. The substrate dependence for P-MLCK was complex, suggesting positive cooperativity,

with an apparent Km of 3-4 μM for phosphoprotein. Activity toward P-MLC at 1 μM was two to three-fold greater than that observed with P-GS or P-MLCK. Since this substrate can be prepared in reasonable quantity and does not have low solubility (as does P-GS) it was used as a model protein substrate for comparison of CaM/CN interaction (fluorescence) and activation (see below).

Previously, it was reported that the Ca^{2+}-dependence for interaction of PDE and D-CaM differed from that for enzyme activation. However, it is difficult to obtain sufficient PDE to carry out a rigorous comparison of the two events and the necessary use of a poorly hydrolyzed, fluorescent substrate, N^6-etheno cyclic AMP, complicates the measurement of fluorescence polarization (interaction). The phosphatase activity of CN can be measured at 0.5 μM with either PNPP or P-MLC under conditions suitable for fluorescence measurements; thus, both interaction and activation can be assayed in the same sample. Furthermore, D-CaM activated CN ~ 85% as well as unmodified CaM with a Ca^{2+}-dependence indistinguishable from that of native CaM, with PNPP as substrate. For either substrate, the K_a for Ca^{2+} appeared to be 26-30 μM in the presence of 5 mM Mg^{2+}, with apparent cooperativity in activation; interestingly higher Ca^{2+} (100-200 μM) decreased activity 30-40%. By contrast, protein interaction in the same samples required far less Ca^{2+} (8-12 μM) with no evidence of cooperativity. This clearly demonstrates a non-identity in the Ca^{2+}- requirements of the two events for protein phosphatase. Based on the fluorescence properties of D-CaM reported previously, the data are consistent with the following hypothesis: The binding of Ca^{2+} to two high affinity sites on CaM is required for the formation of a protein phosphatase/CaM complex, however, this complex remains inactive until a third Ca^{2+} site is occupied. Because of the apparent increase in cooperativity, the intrinsic affinity for Ca^{2+} at this third site may be markedly increased due to complex formation, providing a sensitive mechanism for enzyme activation/deactivation by Ca^{2+}.

2) Immunocytochemical localization of CN and PDE in rat brain and detection of CaM-binding proteins in isolated spleen cells and cultured cells. As previously reported, a purified rabbit IgG fraction which specifically recognizes CaM-dependent PDE was prepared. This high affinity antibody did not react with other forms of PDE, with several CaM-binding proteins (CaM-BPs), nor with any other cytosolic proteins from brain. After initial difficulties, an isolation procedure for anti-CN antibodies from goat plasma was developed with CN coupled to CnBr-activated Sepharose. Prior to purification of this antibody there was substantial non-specific binding and apparent reactivity toward PDE. However, after affinity purification, quantitative ELISA procedures showed < 1% cross-reactivity between PDE and CN antibodies. The affinity purified antibody recognized both the 61 kDa and 18 kDa subunits of the phosphatase and did not react with PDE, MLCK, CaM-dependent protein kinase (CaM-PK) or other proteins present in brain cytosol, by Western-blot analysis.

A question of physiologic importance is whether there is coordinate regulation of several CaM-dependent enzymes in the same cell. Alternatively, cells may not contain many CaM-regulated enzymes but may express only those which are important for specific cell functions. In collaboration with Drs. M.L. Billingsley and C.D. Balaban, specific antibodies were used to study the regional and

subcellular localization of CN and PDE in rat brain slices; in so doing, expression and regulation of these CaM-binding proteins (and the functions they subserve) might be examined. Both CN and PDE (which have their highest concentration in brain) were found exclusively in nerve cells, with no reactivity in glial and other non-neuronal cells. However, CN was found broadly distributed throughout the brain, while PDE was observed in only very few neuronal populations. CN was found in both pre-synaptic and post-synaptic nerves in all regions of the brain; high concentrations were found in all layers of the cerebral cortex, substantia nigra, hippocampus and in selected nuclei of the hypothalamus (e.g., paraventricular nucleus (PVN)). Staining appeared mostly along cell bodies, although dendrites and axons were also stained . The localization of PDE, by contrast, was highly concentrated in the dendrites and somatic cytoplasm of large neurons receiving multiple convergent inputs (i.e., cerebellar Purkinje cells, pyramidal cells of cerebral cortex and hippocampus and in cells of the olfactory cortex). In cerebellum and hippocampus, there was virtually no reaction with many other nerve cells in the same region, such as climbing fibers and hippocampal interneurons and all reactivity was post-synaptic. In some areas prominently stained for CN (such as the PVN) there was no immunoreactivity observed for PDE. Thus, the regional and cellular localization of these two CaM-BPs are clearly different and argues against a concerted Ca^{2+}-dependent regulation of both enzymes in most nerve cells.

At the subcellular level, PDE reactivity appeared to be homogenously distributed in the cytoplasm of cell bodies and dendrites, while CN was often highly concentrated in dense foci, suggesting compartmentalization. Although some diffuse reactivity was observed with CN in cellular cytoplasm, there was a striking punctate distribution along the membranes of cell bodies and proximal dendrites which is consistent with synaptic localization. These immunocytochemical data imply different regulatory roles for CaM-dependent PDE and protein phosphatase activities. PDE may function to control cyclic nucleotide levels in specific nerves which integrate a large amount of synaptic input, thus serving a role in modulating electrochemically summed nerve potentials. CN, on the other hand, may play a general role in Ca^{2+}-regulated phosphoprotein dephosphorylation in nerve cells; its high concentration at synaptic terminals may suggest an important phosphoprotein substrate at these sites. Taken together, the data suggest that Ca^{2+} and CaM can regulate different functions according to the requirements of a particular neuron, and the specific expression of CaM-dependent enzymes and their subcellular localization in such cells may contribute to the unique properties of a given neuron. Moreover, the findings indicate that caution must be exercised when proposing "coordinated" models of regulation by Ca^{2+}/CaM; without direct evidence of co-localization, such models would appear unwarranted.

An examination of CaM-BPs in cultured cells and in cell populations isolated from spleen has been initiated with an emphasis on regulation of their expression after cell "activation". Rat pheochromocytoma (PC-12) cells display phenotypic "differentiation" (neurite outgrowth) upon addition of nanomolar concentrations of nerve growth factor. In the "undifferentiated" state, the content of CaM-BPs appeared to be very low and preliminary purification of this fraction using CaM-Sepharose chromatography was carried out. Analysis of this

enriched fraction by immunological and CaM-binding detection methods (see biotinylated CaM below) indicated the presence of substantial amounts of CN, CaM-PK and the cytoskeletal protein, spectrin; no PDE was detected. Since these cells can be grown in reasonably large quantities, studies of NGF-regulated expression of specific CaM-BPs will be carried out and correlative studies on the enzyme activities (phosphorylation/dephosphorylation) will be undertaken as appropriate.

Several important steps in the process of activation of B and T lymphocytes are Ca^{2+}-dependent. An increase in intracellular Ca^{2+} is known to be an early event in the interaction of cytotoxic T lymphocytes with their target cells. However, little information is available on the biochemical steps involved in such Ca^{2+}-dependent regulation. In collaboration with Dr. M.V. Sitkovsky, whole spleen cells and purified fractions of thymocytes, B and T cells from mice have been examined for the presence of CaM BPs, PDE, and CN in fashion similar to that detailed for PC-12 cells (see above); preliminary results indicate that, by far, the predominant CaM BP in such cells is CN, albeit in relatively low abundance compared with brain tissue. Indeed, neither PDE or other CaM-BPs have been detected. The phosphatase appears to be greatly enriched in B cells, and in this cell type, the molecular weight of the catalytic peptide (59 kDa) is slightly lower than that seen in thymocytes or bovine brain (61 kDa); this may represent proteolysis (although no other degradation products were observed) or perhaps a post-translational modification of the phosphatase. Examination of the various CaM BPs after cell activation will be carried out using both isolated spleen cells and clonal cell lines which exhibit phenotypic responses characteristic of that cell type.

3) Development of a non-radioactive CaM-BP detection method: Detection of CaM-regulated proteins with iodinated CaM overlay methods presents difficulties in the preparation and use of the derivative, and extensive washing of transfer media is required to reduce background radioactivity. In collaboration with Dr. M.L. Billingsley, a method was developed for the biotinylation of CaM and its subsequent use for CaM-BP detection using avidin coupled to peroxidase. Biotinylation of CaM ($\sim$ 2 mol biotin/mol CaM), which occurs primarily through modification of lysine groups, reduced the biological activity of CaM only slightly; CN phosphatase activity was activated to $\sim$ 85% that seen with native CaM. After passive (slot-blot) and electrophoretic (Western blot) adsorption of purified CN, biotinylated CaM (bioCaM) was able to detect as little as 10 ng CN. As expected, only the 61 kDa CaM-binding subunit was detected. When crude cytosolic or membrane extracts from heart and brain were analyzed by Western blots followed by bioCaM incubation, only peptides with molecular weights characteristic of known CaM-BPs were detected. Also, in a partially purified fraction from brain, bioCaM was reactive only with material before chromatography on CaM-Sepharose (i.e., depletion of CaM-BPs from the fraction eliminated bioCaM response). When two-dimensional electrophoresis of CN was carried out, several closely-related spots, with pIs of 5.2 - 5.6 were visualized after bioCaM treatment; these may reflect isoforms of the catalytic subunit of CN. The speed and sensitivity of the bioCaM method should make it possible to screen many crude fractions for putative CaM-BPs, even when material is in limited

quantity. Its use, in conjunction with two dimensional electrophoresis (or other "mapping" techniques) should provide a powerful approach to analysis of microheterogeneity and functional binding domains among CaM-BPs.

Significance to Biomedical Research and the Program of the Institute: The effects of many biochemical regulators, such as hormones, on mammalian cells are mediated by altering the rates of synthesis and/or degradation of cyclic nucleotides and by the phosphorylation/dephosphorylation of certain cellular proteins. Investigation of the properties and distribution of the enzymes regulating these procedures is important for the understanding of normal and pathologic cellular activity and may permit design of rational therapeutic approaches. The well-established significance of CaM in control of diverse cellular functions makes the elucidation of its mechanism of action important for the understanding of calcium-regulated processes. The development of new biochemical methods (e.g., novel affinity chromatography approaches) may provide useful tools for investigation of basic biochemical and enzymologic process.

Proposed course: To investigate potential enzymatic relationships between phospho and dephospho forms of PDE, CN and CaM PK and/or their substrates. To document the content and distribution of several CaM-binding proteins (CN, PDE, CaM PK, MLCK) in mammalian tissues and in cultured cells, and to investigate developmental or regulatory changes in their content. To develop cell-free translation methods and oligonucleotide probes for investigation of the expression of PDE and CN in such systems.

Publications:

Vaughan, M. and Kincaid, R.L.: Mechanism of activation of cyclic nucleotide phosphodiesterase by calmodulin: Utilization of novel biologically active derivatives of calmodulin to probe mechanisms of Ca^{2+}-dependent interaction and enzyme activation. Sixth US-USSR Symposium on Myocardial Metabolism, in press.

Kincaid, R.L. and Vaughan, M.: Molecular and regulatory properties of calmodulin-dependent phosphodiesterase form brain. Calcium and Cell Regulation, New York, Raven Press, in press.

Billingsley, M.L., Kincaid, R.L., and Lovenberg, W.: Stoichiometric methylation of calcineurin by protein O-carboxylmethyltransferase and its effects on calmodulin-stimulated phosphatase activity. Proc. Natl. Acad. Sci. (U.S.A.), in press, Sept. 1985.

Billingsley, M.L., Kincaid, R.L., Wolf, M.E., Roth, R.H., Lovenberg, W. and Balaban, C.D.: Is there a function for protein carboxylmethylation in the nervous system S-Adenosyl Methionine as a Basis for Drug Design. Clifton, New Jersey, Humana Press, in press, 1985.

Kincaid, R.L., Stith-Coleman, I.E., and Vaughan, M.: Proteolytic activation of calmodulin-dependent cyclic nucleotide phosphodiesterase, J. Biol. Chem., in press, July, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00622-08 CM

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Cyclic Nucleotide Metabolism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)* Departments of Pharmacology and Medicine, University of Virginia School of Medicine, Charlottesville, VA (E.L. Hewlett); Molecular Disease Branch, NHLBI (J.O. Osborne, Jr.); NATO Fellowship (P. Bruni); Department of Biochemistry, North Texas State U. and Texas College of Osteopathic Medicine, Denton, Texas

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Laboratory of Cellular Metabolism

SECTION
Molecular Mechanisms

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.1	2.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1) cAMP and cGMP, are critical intracellular messengers, under the control of exogenous agents. In N1E-115 cells, muscarinic agonists affect both cAMP and cGMP, causing suppression and elevation, respectively. Muscarinic suppression of cAMP levels was not blocked by cAMP phosphodiesterase inhibitors, and thus results from inhibition of adenylate cyclase and not activation of phosphodiesterase. Inhibitory effects of muscarinic agents on cAMP are mediated by a guanyl nucleotide-binding protein, Gi; the mechanism by which they influence cGMP generation is unclear. Pertussis toxin, by catalyzing the ADP-ribosylation of Gi, reduced the muscarinic suppression of basal and prostaglandin-stimulated cAMP. The toxin did not affect muscarinic agonist-dependent cGMP levels, consistent with the hypothesis that Gi is not involved in this pathway. 2) NAD:arginine ADP-ribosyltransferases, previously identified in animal tissues, catalyze reactions similar to bacterial toxins, such as choleragen, which cause activation of adenylate cyclase. Kinetics of the reactions catalyzed by choleragen and an erythrocyte ADP-ribosyltransferase were examined with agmatine, an arginine analogue, as the ADP-ribose acceptor. The data were most consistent with a random, rapid equilibrium, sequential mechanism; binding of either NAD or agmatine had a negative effect on the subsequent binding of the other substrate. 3) NAD:arginine ADP-ribosyltransferases catalyze the ADP-ribosylation of arginine and proteins. An enzymatic activity was identified in erythrocytes that cleaved ADP-ribosylarginine to ADP-ribose and arginine. The reaction was stimulated by divalent cations (e.g., Mg^{2+}) and thiols. A product of the reaction, arginine, was, in the presence of NAD, ADP-ribosylated by choleragen and erythrocyte transferase. Since degradation of ADP-ribosylarginine generates an arginine moiety that is a substrate for the transferases, it appears that ADP-ribosylation may be a reversible covalent modification.

Project Description:

Objectives: To define mechanisms for regulation of cyclic nucleotide metabolism. cAMP and cGMP content of animal cells is regulated in part by external agents, such as hormones, drugs, and bacterial toxins. Control over cAMP synthesis appears to be exerted through the hormone-sensitive adenylate cyclase system. Cyclase activity is regulated by stimulatory and inhibitory agonists operating through their respective cell surface receptors; these receptors are linked to the catalytic unit through different GTP-binding proteins, termed G_s and G_i respectively. G_s and G_i are similar in structure and possess GTP-binding subunits $G_{s\alpha}$ and $G_{i\alpha}$ of 45,000 and 41,000 Da, linked to a common heterodimer, $G_{\beta\gamma}$ of 35 kDa (G_β) and $\sim$ 10 kDa (G_γ). These proteins either directly or indirectly alter catalytic unit activity. Studies on the activation of adenylate cyclase by the bacterial toxins choleragen and pertussis toxin have defined the roles of G_s and G_i in the control of catalytic unit activity; these toxins exert their effects on cells by catalyzing the transfer of the ADP-ribose moiety of NAD to critical amino acid residues on these regulatory proteins. Choleragen catalyzes the ADP-ribosylation of $G_{s\alpha}$ at what is probably an arginine residue. A 41,000 Da subunit of G_i is the target of pertussis toxin (islet-activating protein), a protein product of Bordetella pertussis, an etiologic agent of whooping cough. ADP-ribosylation leads to inactivation of the protein; agents such as opiates, muscarinic agonists and α_2-adrenergic agonists no longer inhibit adenylate cyclase. In some cells, muscarinic agonists may depress cAMP content by stimulating hydrolysis through activation of a cyclic nucleotide phosphodiesterase; in this case, pertussis toxin does not block muscarinic suppression of cAMP content. A direct coupling of cell surface receptors to particulate or soluble guanylate cyclases has not been established; muscarinic agonists may, however, also cause an increase in cGMP. Thus, it would appear that this agent operates through both cyclic nucleotide systems. In N1E-115 cells, muscarinic receptors suppress cAMP content and increase that of cGMP. These cells were used as a model system to study the kinetics of muscarinic agonist effects on cAMP and cGMP. Pertussis toxin was used to examine the role of G_i in carbamylcholine action on both cyclic nucleotides.

These studies and other reports have defined the importance of toxin-catalyzed mono-ADP-ribosylation in the regulation of adenylate cyclase. Animal tissues appear to possess endogenous ADP-ribosyltransferases that catalyze reactions similar to the toxins. Prior studies in the laboratory defined a family of NAD:arginine ADP-ribosyltransferases in turkey erythrocytes. Similar enzymatic activities were reported in rabbit skeletal muscle, chicken liver nuclei and Xenopus tissues. To further define the mechanism of action of these enzymes, the kinetics of ADP-ribosylation were examined with a purified erythrocyte ADP-ribosyltransferase.

If ADP-ribosylation were to serve a regulatory role, then it might be expected that specific enzymes exist which remove the ADP-ribose moiety, regenerating, intact, the acceptor group. To examine this possibility animal cells and tissues were surveyed for an ADP-ribosylarginine cleavage activity.

Methods Employed: 1) Assays: (A) NAD glycohydrolase and ADP-ribosyltransferase assays were performed by modifications of methods developed in this laboratory. (B) ADP-ribosylarginine cleavage enzyme was assayed by a published procedure developed in the laboratory using ADP-ribosyl-[^{14}C]arginine as a model substrate. A product of the reaction, [^{14}C]arginine, was resolved from the substrate by chromatography on a phenylboronate polyacrylamide resin.

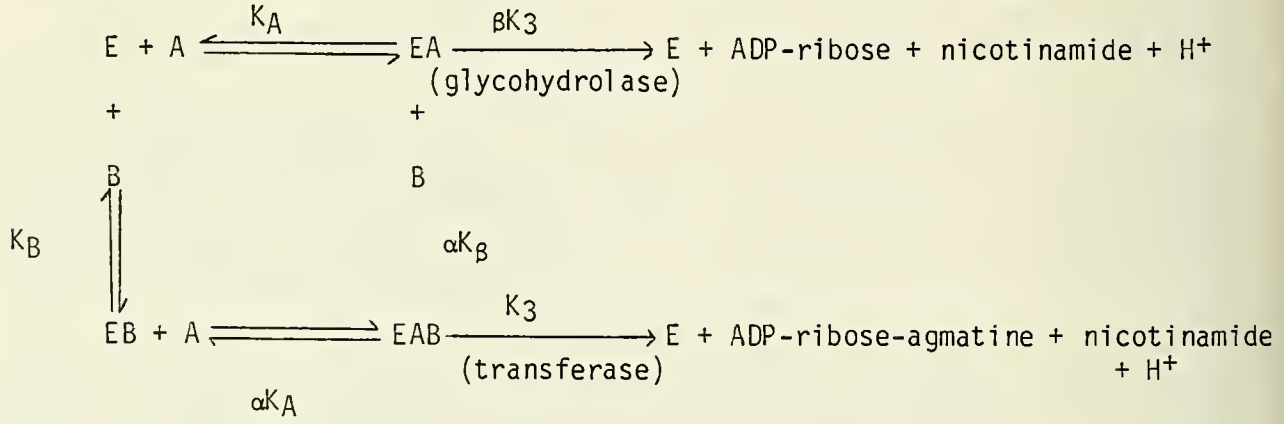
2) Enzyme Purification: NAD:arginine ADP-ribosyltransferase was purified from turkey erythrocyte supernatant by methods developed in the laboratory.

Major Findings: Role of G_i in the effects of carbamylcholine on cAMP and cGMP. As noted previously, in N1E-115 neuroblastoma cells, carbamylcholine, a muscarinic agonist, increased cGMP over 15-fold, and decreased basal and PGE_1-stimulated cAMP content. Reduction by carbamylcholine of PGE_1-stimulated cAMP levels was observed in the presence of the phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine, Ro-20-1724 or both. In contrast to the stimulatory effects of PGE_1 on cAMP which were immediate, the carbamylcholine-induced decrease in basal and PGE_1-stimulated cAMP exhibited a delay. The delay in carbamylcholine inhibition was independent of the extent of adenylate cyclase activation. Although basal cAMP content was suppressed within 30 sec after addition of carbamylcholine, inhibition was not maximal for at least 2 min following agonist addition; the delay was similar in cells exposed to PGE_1 for 10 min prior to carbamylcholine but could be eliminated by incubation of the cells with muscarinic cholinergic agonist for 5 min prior to addition of prostaglandin. N1E-115 neuroblastoma cells contain a 41,000 Da membrane protein believed to be a component of the inhibitory GTP-binding protein of adenylate cyclase that is ADP-ribosylated by pertussis toxin. Incubation of the cells with pertussis toxin prior to the addition of carbamylcholine reduced the maximal extent of inhibition of cAMP content and prevented the [^{32}P]ADP-ribosylation of a 41,000 Da protein by toxin and [^{32}P]NAD in membrane preparations from these cells. Incubation of cells with pertussis toxin, however, did not significantly alter the dose response curve for carbamylcholine effects on cGMP. Even high concentrations of carbamylcholine, effective in stimulating cGMP, had minimal effects on cAMP content in toxin-treated cells; thus, ADP-ribosylation of G_i converts the adenylate cyclase but not the guanylate cyclase system to an agonist-insensitive state.

Effects of muscarinic cholinergic agents on cAMP content are believed to proceed by two mechanisms. The first pathway involves activation of the ligand receptor complex and G_i, leading to decreased activity of the adenylate cyclase catalytic unit and thus, reduced cAMP synthesis. The second mechanism for decreasing cAMP content by muscarinic agonists involves activation of cyclic nucleotide phosphodiesterase(s), leading to increased cAMP degradation. Pertussis toxin sensitivity of the carbamylcholine-inhibited cAMP accumulation may be used to distinguish events dependent on adenylate cyclase from those acting through phosphodiesterase. Muscarinic receptor-mediated inhibition of cellular cAMP levels thorough adenylate cyclase is sensitive to pertussis toxin, whereas attenuation of cAMP accumulation through phosphodiesterase activation is not subject to toxin inhibition. Since the presence of phosphodiesterase inhibitors

such as 3-isobutyl-1-methylxanthine or Ro-20-1724 did not reduce carbamylcholine effectiveness whereas toxin blocked inhibition by agonists it would appear that the effects of carbamylcholine are mediated in N1E-115 cells predominantly through adenylate cyclase inhibition rather than by phosphodiesterase activation.

2) Kinetic studies on choleragen and NAD:arginine ADP-ribosyltransferase. ADP-ribosylation is a covalent modification of proteins catalyzed by viral, bacterial, and animal enzymes; the ADP-ribose moiety of NAD is transferred to either an acceptor protein or to another ADP-ribose moiety to yield a protein-linked monomer or polymer. These synthetic reactions are catalyzed by two classes of enzymes: (a) mono(ADP-ribose)transferases, which catalyze the mono-ADP-ribosylation of proteins, and (b) poly(ADP-ribose)synthetases, which catalyze the initial ADP-ribosylation of protein and subsequent chain elongation. The importance of the mono-ADP-ribosylation reaction has been best documented for bacterial toxins such as choleragen that exert their effects on cells by modifying critical enzymes. Among the ADP-ribosyltransferases endogenous to animal cells are those that catalyze the formation of ADP-ribosylarginine. These enzymes, which also modify proteins, presumably using a guanidino moiety as an ADP-ribose acceptor, have been purified from turkey erythrocytes, chicken liver and rabbit muscle. A family of NAD:arginine ADP-ribosyltransferases has been identified in turkey erythrocytes and localized in the nuclear, cytosolic, and membrane fractions. These transferases differ in physical, kinetic, and regulatory properties. Choleragen also possesses NAD:arginine ADP-ribosyltransferases activity; toxin-catalyzed activation of the adenylate cyclase system is believed to result from modification of an arginine residue on a cyclase regulatory protein. The kinetic mechanism for choleragen and a highly purified NAD:arginine ADP-ribosyltransferase from turkey erythrocytes was investigated in the presence and absence of the product inhibitor, nicotinamide, using agmatine, an arginine analogue, as an acceptor. To obtain accurate estimates of kinetic parmeters, the transferase and glycohydrolase reactions were monitored simultaneously by using a mixture of [adenine-U-^{14}C] NAD and [carbonyl-^{14}C]NAD as tracers. The kinetic analyses supported a rapid equilibrium model for both the avian NAD:arginine ADP-ribosyltransferase and the enzymatically active subunit of cholera toxin.



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In this model A and B correspond to NAD and agmatine and the equilibrium dissociation constants are given by K_A and K_B, respectively. The factor β is equal to the ratio of maximal velocities of the glycohydrolase and transferase reactions. Both NAD and ADP-ribose acceptors bind randomly to the transferase or choleragen; binding of either NAD or agmatine has a negative effect on the subsequent binding of the other substrate. This mechanism for choleragen differs from one proposed earlier which postulated an ordered sequential mechanism in which the first substrate to bind was NAD. The present studies are consistent with the hypothesis that, under our assay conditions, using agmatine as the ADP-ribose acceptor, the substrates can bind to choleragen in random order. The observation that binding of one substrate lowered the affinity for the second substrate led us to believe that we might be able to differentiate kinetically between rapid equilibrium random and steady-state ordered mechanisms by product inhibition studies. The binding of the competitive inhibitor nicotinamide changed the affinity for agmatine and vice versa, as was found for NAD and agmatine; the system could be described using an inhibitor interaction parameter, γ. If $\gamma = 1$, then the apparent inhibition constant would vary with agmatine concentration. For ET γ was close to 1, and therefore kinetic studies with nicotinamide could not be used to differentiate between rapid equilibrium random and steady-state ordered mechanisms. The inhibitor interaction parameter for choleragen, 5.2, indicated that the binding of the acceptor agmatine decreased the affinity for nicotinamide by approximately half an order of magnitude; the apparent inhibition constant for nicotinamide increased with increasing concentrations of agmatine. The combined results for choleragen were most consistent with a random rapid equilibrium mechanism.

Under the framework of the rapid equilibrium mechanism, one obtains the "true," i.e., unperturbed, Michaelis constants for both substrates as well as the factor by which binding of one substrate changes the affinity for the second substrate. This factor, α, must be the same for both substrates, since this model assumes rapid equilibrium binding of both substrates and changes in free energy must be independent of path. For the erythrocyte transferase and CT, α was found to be approximately 3.5 with agmatine as the acceptor and therefore binding of NAD or agmatine results in a greater than 70% decrease in the affinity for the second substrate. Conceivably, in the presence of the physiological acceptor, which has not yet been identified, α may differ from the value found for agmatine. To some extent, variation in α may enhance substrate specificity such that certain arginine residues or guanidino compounds are more readily ADP-ribosylated. Although choleragen appears to be able to use a variety of proteins and guanidino compounds in vitro, in intact cells, it appears that toxin preferentially ADP-ribosylates two membrane proteins of 42 kDa and 47 kDa. Current studies are directed at determining whether α may be critical to the regulation of the roles of ADP-ribosylation of potential acceptor substrates.

3) Identification of an ADP-ribosylarginine cleavage enzyme. Animal cells contain a family of NAD:arginine ADP-ribosyltransferases that catalyze the mono-ADP-ribosylation of arginine and proteins. These NAD:arginine ADP-ribosyltransferases under physiological conditions do not appear to catalyze the degradation of the products, ADP-ribose-arginine and ADP-ribose-protein. We therefore

investigated the possibility that a different family of enzymes exists that cleaves the ADP-ribose-arginine linkage. In extracts from Simian virus 40-transformed mouse fibroblasts (SVT2), ADP-ribose-protein, synthesized by an NAD:arginine ADP-ribosyltransferase from erythrocytes, was degraded by endogenous enzymes to release compounds tentatively identified as ADP-ribose and phosphoribose, although other products were formed consistent with proteolytic digestion to yield an ADP-ribose-(amino acid)peptide conjugate(s). To study the reversal of ADP-ribosylation, it was decided to examine the degradation of a model compound, ADP-ribosylarginine, and thus to circumvent the problems inherent in characterization of the products of a more complex ADP-ribose-protein degradation. Since turkey erythrocytes were the source of a family of NAD:arginine ADP-ribosyltransferases, responsible for the synthetic reaction, this tissue was examined for the presence of ADP-ribosylarginine cleavage enzymes.

An enzymatic activity was partially purified from erythocytes that catalyzed the degradation of ADP-ribosyl-[^{14}C]arginine synthesized by a salt-activated NAD:arginine ADP-ribosyltransferase, resulting in the release of a radiolabeled compound that was characterized chromatographically and by amino acid analysis as arginine. This putative arginine product was converted in a reaction dependent on NAD and the NAD:arginine ADP-ribosyltransferase to a compound exhibiting properties characteristic of ADP-ribosylarginine. Action of cleavage enzyme on [adenine-U-^{14}C]ADP-ribosylarginine resulted in the release of a radiolabeled compound that behaved chromatographically like [adenine-U-^{14}C]ADP-ribose. Degradation of ADP-ribosylarginine by the partially purified cleavage enzyme was enhanced by dithiothreitol and $MgCl_2$. Activation was maximal with 5-10 mM dithiothreitol and 5-10 mM $MgCl_2$. These studies are consistent with the hypothesis that the Mg^{2+}- and dithiothreitol-stimulated cleavage of ADP-ribosylarginine resulted in the formation of ADP-ribose and arginine. Since degradation of ADP-ribosylarginine appears to generate an arginine moiety that is a substrate for the NAD:arginine ADP-ribosyltransferase, it appears that ADP-ribosylation may be a reversible modification of proteins.

Significance to Biomedical Research and the Program of the Institute: The pulmonary and cardiovascular systems are affected under physiological and pathological conditions by extracellular agents such as hormones and toxins. The lung, in particular, is exposed through the tracheobronchial tree to a variety of bacterial and toxic agents. A substantial number of these agents exert their effects by altering the steady-state levels and localizations of cyclic nucleotides within the cell. Some bacterial toxins exert their effects on cyclic nucleotides by catalyzing the specific covalent modification of critical regulatory proteins, thereby altering the ability of these proteins to function normally. A number of pharmacological agents have been in use which override aberrant physiological control to the benefit of the patient; these agents have been designed to interact with specific cellular receptors, and thus to alter cyclic nucleotide levels. By using cultured cells, model systems and purified preparations, it may be possible to define the factors critical to cyclic nucleotide metabolism. These models can then be used to understand the controls which operate in the more complex pulmonary and cardiovascular systems.

Proposed Course: (a) To define further the regulation of guanyl nucleotide-binding proteins by hormones and drugs (b) To define the effects of toxin-catalyzed ADP-ribosylation on activity of the guanyl nucleotide-binding proteins; (c) To investigate the regulation of ADP-ribosylarginine cleavage enzyme activity in animal cells.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00625-07 CM

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

ADP-Ribosylation of Transducin by Pertussis Toxin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Robert E. West, Jr., Ph.D.	Staff Fellow	CM, NHLBI
Others:	Joel Moss, M.D., Ph.D.	Head, Section on Molecular Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS *(if any)* Division of Biochemistry and Biophysics, Office of Biologics Research and Review, Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Maryland (Dr. Darrell Liu).

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hormonal control of adenylate cyclase is mediated by GTP-binding proteins, stimulation via Gs, inhibition via Gi. Cholera toxin and pertussis toxin disrupt hormonal control of adenylate cyclase by ADP-ribosylating Gs and Gi, respectively. GTP-binding proteins appear to be involved in other transmembrane signalling events as well. One of these, transducin, and retinal GTP-binding protein that couples the light receptor rhodopsin to a cGMP phosphodiesterase, serves as a substrate for cholera toxin and pertussis toxin. Because transducin shares functional and structural homology with other G proteins it is useful for the study of the toxins' mechanisms. In these studies, we have determined the site of pertussis toxin catalyzed ADP-ribosylation of transducin.

Pertussis toxin catalyzed the incorporation of radiolabel from [U-14C-adenine]-NAD into the α subunit of holotransducin from bovine retinas. Maximal incorporation was 0.3 mol ADP-ribose/mol transducin. A tryptic peptide containing ADP-ribose was purified by sequential chromatography on TSK-DEAE, phenylboronate polyacrylamide and Ultrapore C3. The amino acid content was analyzed and the peptide sequence determined to be glu-asn-leu-lys-asp-x-gly-leu-phe, in agreement with the amino acid analysis which included, in addition, cysteine. This corresponds to the recently published transducin cDNA sequence glu-346 through phe-354 which shows residue 351 to be cysteine. HPLC of the acetic acid eluate of the sequencing resin showed two radioactive species, one eluting between NAD and ADP-ribose, presumably ADP-ribosyl cysteine, and the other coincident with 5'-AMP. Amino acid analysis of the eluate disclosed the presence of cysteine. We conclude that cys-351, not an asparagine as previously reported is the site of pertussis toxin-catalyzed ADP-ribosylation of transducin. Because of transducin's sequence homology with Gs and Gi, this is most likely the modification site in these proteins as well. This cysteine is apparently critical for the coupling of inhibitory receptors to adenylate cyclase.

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Project Description:

Objectives: Hormonal control of adenylate cyclase is mediated by receptors for stimulatory and inhibitory ligands that act through regulatory proteins, termed G_s and G_i, respectively. Since G_s and G_i are present in minute amounts in animal tissues, investigation of their properties has been limited. However, transducin, a protein that is structurally and functionally similar to G_s and G_i, is present in reasonable quantities in retinal rod outer segments (ROS) and is easily isolated. Transducin, G_s, and G_i are heterotrimers, each consisting of subunits designated α (39, 45, and 41 k Da, respectively), β (36, 35, and 35 k Da, respectively), and γ (5-10 k Da). Choleragen activates adenylate cyclase by catalyzing the ADP-ribosylation of $G_{s\alpha}$, resulting in inhibition of its GTPase activity which is normally increased by stimulatory hormones. ADP-ribosylation by choleragen of the α subunit of transducin (T_α) in ROS, results in decreased GTPase activity. Pertussis toxin (islet-activating protein) ADP-ribosylates $G_{i\alpha}$, thereby preventing hormonal inhibition of adenylate cyclase and stimulation of GTPase activity of $G_{i\alpha}$. The objective of this project was to determine the site of pertussis toxin-catalyzed ADP-ribosylation on transducin.

Methods Employed: Transducin was purified and ADP-ribosylated by published methods. Tryptic digestion and purification of the ADP-ribosylated peptide was performed according to Manning et al. Automated Edman degradation was performed in an applied Biosystem gas phase sequencer 470A; degradation was carried out in the presence of Polybrene. Phenylthiohydantoin amino acids were identified by high pressure liquid chromatography. Amino acid analysis was performed on performic acid oxidized peptides, hydrolyzed in methanesulfonic acid/tryptamine. The hydrolysates were analyzed according to Spackman et al. with a single column system.

Major Findings: Pertussis toxin catalyzes the transfer of ADP-ribose from NAD to the guanine nucleotide-binding regulatory proteins, G_i, G_o, and transducin. Based on a partial amino acid sequence for a tryptic peptide of ADP-ribosylated transducin, asparagine had been characterized as the site of pertussis toxin-catalyzed ADP-ribosylation. Subsequently, cDNA data for the α subunit of transducin indicated that the putative asparagine residue was, in fact, not present in the protein. To determine the amino acid that served as the ADP-ribose acceptor, radiolabel from [U-^{14}C-adenine]NAD was incorporated, in the presence of pertussis toxin, into the α subunit of transducin (0.3 mol/mol). An ADP-ribosylated, tryptic peptide was isolated by DEAE and phenylboronate polyacrylamide chromatography. The peptide was further purified by reverse-phase chromatography on an Ultrapore RPSC C3 column. The major peak of radioactivity was fully sequenced by automated Edman degradation. The amino acid sequence, glu-asn^{347}-leu-lys-asp^{350}-x-gly^{352}-leu-phe, corresponds to the cDNA sequence coding the carboxyl terminal nonapeptide, glu^{346}-phe^{354}, which includes by cDNA sequence cysteine at position 351. Amino acid analysis of the nonapeptide confirmed the presence of cysteic acid. The sequencing study was consistent with the proposal that neither asn^{347} nor asp^{350} was modified; residue^{351} adhered to the sequencing resin. Cysteine, the missing residue,

was eluted from the sequencing resin with acetic acid along with 76% of the peptide-associated radioactivity half of which, presumably ADP-ribosylcysteine, eluted from an anion exchange column between NAD and ADP-ribose; the other half had a retention time corresponding to 5'-AMP. We conclude that cys^{351} and not asn^{347} or asp^{350} is the site of pertussis toxin-catalyzed ADP-ribosylation in transducin.

Among the ADP-ribosyltransferases whose sites of action have been ascertained, pertussis toxin, transferring to a cysteine, is unique. Glutamate is the site of nuclear poly-ADP-ribosylation. Cholera toxin, E. coli heat-labile enterotoxin, and the mono-ADP-ribosyltransferases native to turkey erythrocytes and chicken liver nuclei modify a substrate arginine; diphtheria toxin and, apparently, Pseudomonas exotoxin A and an enzyme native to bovine liver transfer ADP-ribose to diphthamide, a modified histidine apparently restricted to EF-2. The limited occurrence of diphthamide may explain, in part, the specificity of diphtheria toxin for EF-2. The determinants of specificity for cholera toxin, E. coli heat-labile enterotoxin and the vertebrate transferases are unclear since all of these will transfer ADP-ribose to arginine and other guanidino compounds in solution as well as to their native substrates. The degree of specificity of pertussis toxin for the ADP-ribose acceptor site remains to be seen. Given the structural similarities among the pertussis toxin substrates, $G_{i\alpha}$, $G_{o\alpha}$, and T_{α}, and substantial sequence homologies between T_{α} and $G_{o\alpha}$, including the carboxyl termini of both, where pertussis toxin-catalyzed ADP-ribosylation occurs, cysteine likely serves as the ADP-ribose acceptor in the pertussis toxin-catalyzed modification of all three proteins.

Significance to Biomedical Research and the Program of the Institute: Adenylate cyclase is a ubiquitous enzyme that plays a crucial role in mediating the actions of a variety of hormones and drugs. Our study has provided new information about its regulation and has suggested a means for identification and purification of endogenous tissue NAD: ADP-ribosyltransferases that, like pertussis toxin, modify specific cysteine residues in proteins.

Proposed course: 1) Develop an assay for pertussis toxin ADP-ribosyltransferase activity. (2) Determine whether as with cholera toxin, there are enzymes in higher organisms with mechanisms analogous to that of pertussis toxin. (3) Determine the reversibility of transducin ADP-ribosylation. (4) Determine the importance of the ADP-ribosylated sequence for G-protein function.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00627-07 CM

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

GTP-Binding Proteins and Adenylate Cyclase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Su-Chen Tsai, Ph.D.	Research Chemist	CM, NHLBI
Others:	Yasunori Kanaho, Ph.D.	Visiting Fellow	CM, NHLBI
	Joel Moss, M.D., Ph.D.	Head, Section on Molecular Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Adenylate cyclase systems consist of inhibitory and stimulatory receptors linked to a catalytic unit through inhibitory and stimulatory proteins, Gi and Gs, respectively. Similarly, the retinal photon receptor rhodopsin is linked with a phosphodiesterase through transducin (T), which is similar to Gs and Gi. Each has a guanyl nucleotide-binding α subunit (45 kDa Gs, 41 kDa Gi, and 39 kDa T) and β, γ subunit (35 and 10 kDa). Gs and Gi can be activated by NaF with AlCl3 and by decavadanate, as well as by agonists acting through specific receptors. The effects of NaF with AlCl3 and decavanadate on transducin were investigated in a reconstituted system consisting of the purified subunits of transducin (Tα, Tβγ) and rhodopsin in phosphatidylcholine vesicles. NaF noncompetitively inhibited the GTPase activity of Tα in a concentration- and time-dependent manner. Inhibition by NaF was enhanced synergistically by AlCl3 which alone only slightly inhibited GTPase activity. None of the other anions tested reproduced the effect of fluoride. Fluoride inhibited [3H]GppNHp binding to Tα and release of bound GDP. Vanadate (decameric) inhibited [3H]GTP binding to Tα and GTP hydrolysis in a concentration-dependent manner with maximal inhibition of ∿90% at 3-5 mM. Vanadate also inhibited release of bound GDP. The ADP-ribosylation of Tα by pertussis toxin and binding of Tα to rhodopsin, both of which are enhanced in the presence of Tβγ, were inhibited by NaF with AlCl3 and by vanadate. These findings are consistent with the conclusion that NaF plus AlCl3 or vanadate can cause the dissociation of Tα from Tβγ , resulting in the inhibition of GDP-GTP exchange and thereby GTP hydrolysis. Adenylate cyclase activation and inhibition presumably result from similar effects of NaF plus AlCl3 and vanadate on Gs and Gi.

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Project Description

Objectives: To define the regulation of the hormone-sensitive adenylate cyclase system. The hormone-sensitive adenylate cyclase system is critical to the regulation of cellular processes by hormones, toxins, and drugs. The system consists of stimulatory and inhibitory receptors linked through different guanyl nucleotide-binding regulatory proteins to a catalytic unit. It appears to be analogous to the retinal rod outer segment rhodopsin-transducin-cGMP phosphodiesterase complex, where rhodopsin is the photon receptor and transducin the guanyl nucleotide-binding coupling protein. These guanyl nucleotide-binding proteins are heterotrimers consisting of α, β, and γ subunits. The α subunits bind guanyl nucleotides and catalyze GTP hydrolysis; the $\beta\gamma$ subunits in the presence of receptor promote GTP hydrolysis by the α subunit. The goals of the project are to define the mechanisms of interaction of the α and $\beta\gamma$ subunits with each other and with receptors.

Methods Employed: (a) Protein Purification: Transducin and rhodopsin were purified by published procedures. The α and $\beta\gamma$ subunits of transducin were isolated by Blue Sepharose CL-6B.

(b) Enzyme Assays: GTP hydrolysis, guanyl nucleotide-binding, and GDP release were determined by published procedures. ADP-ribosylation of the β subunit by pertussis toxin was performed by methods developed in the laboratory.

Major Findings: In the adenylate cyclase system, inhibitory and stimulatory agonists affect cAMP synthesis by binding to specific receptors which are coupled to the catalytic unit through inhibitory and stimulatory guanyl nucleotide-binding proteins, termed G_i and G_s, respectively. G_s and G_i are heterotrimers consisting α, β, and γ subunits. The α subunits, 41 kDa in G_i and nominally 42-47 kDa in G_s, bind GTP and possess intrinsic GTPase activity. The β and γ subunits (36 and $\sim$ 10 kDa) appear to be identical in G_i and G_s. Activation of G_s by the agonist-receptor complex is believed to result from dissociation in the presence of the inactive, $G_{s\alpha}\cdot G_{\beta\gamma}$, to yield $G_{s\alpha}$.GTP, which directly stimulates the catalytic unit. Activation is terminated by hydrolysis of bound GTP and reassociation of $G_{s\alpha}$.GDP with $G_{\beta\gamma}$. The mechanism of inhibition by G_i is less clear, although, like G_s, G_i is activated by dissociation of the α and $\beta\gamma$ subunits.

In the retinal light-activated cGMP phosphodiesterase system, transducin couples the photoexcitation of rhodopsin to the stimulation of a cGMP phosphodiesterase in retinal rod outer segments. Transducin is a heterotrimer with α, β, and γ subunits of 39, 36, and $\sim$ 8 kDa respectively. T_α is a GTP-binding protein responsible for the activation of the cGMP phosphodiesterase while $T_{\beta\gamma}$ serves in coupling T_α to photolyzed rhodopsin. The interaction of T_α with photolyzed rhodopsin stimulates release of GDP and binding of GTP, with resulting dissociation of $T_{\beta\gamma}$ from T_α.GTP, which, in turn, activates the phosphodiesterase. The hydrolysis of bound GTP by T_α terminates the active state and leads to formation of the T_α.GDP.$T_{\beta\gamma}$ complex. Photolyzed rhodopsin by promoting GTP-GDP exchange increases GTP hydrolysis by T_α.

Transducin and G_i share functional and structural similarities. Manning and Gilman demonstrated that the respective α and β subunits of transducin and G_i have similar protease digestion patterns and amino acid composition. In the presence of photolyzed but not dark rhodopsin, T_α and $G_{i\alpha}$, when reconstituted with either $G_{\beta\gamma}$ or $T_{\beta\gamma}$, exhibited GTPase activity. Both T_α and $G_{i\alpha}$ served as substrates for ADP-ribosylation by pertussis toxin, which decreasedlight and hormone-stimulated GTP hydrolysis, respectively. The toxin-catalyzed ADP-ribosylation abolished hormonal inhibition of cyclase and light-stimulation of the phosphodiesterase.

Adenylate cyclase can be both activated and inhibited by NaF acting through G_s and G_i, respectively. Sternweis and Gilman showed that Al^{3+} was required for activation of G_s by F^-. In view of the similarities between G_i and transducin, we investigated the effects of NaF and $AlCl_3$ on transducin function. Like NaF, vanadate and related ions have also been reported to both activate and inhibit adenylate cyclase. The effects of vanadate on transducin function in a reconstituted system with rhodopsin were also investigated.

GTPase Activity: As reported by Fung, the GTPase activity of T_α was observed only after reconstitution with $T_{\beta\gamma}$ and photolyzed rhodopsin. In our assays with 5 μM substrate, a maximum of 40% of GTP was hydrolyzed in 10 min at 30°C, i.e., GTPase activity was ca. 0.3 pmol/min/pmol of T_α. Under these conditions NaF inhibited GTPase activity in a concentration-dependent manner and inhibition was enhanced by 20 μM $AlCl_3$. Inhibition was maximal with 10 mM NaF in the absence and 2.5 mM NaF in the presence of $AlCl_3$. Although $AlCl_3$ alone inhibited GTPase relatively little, it markedly increased inhibition by 2.5 mM NaF with a maximal effect at ∿ 20 μM and half-maximal effect at 7.5 μM. Inhibition by NaF was noncompetitive with an apparent K_i of 5.3 mM in the absence and 0.5 mM in the presence of 20 μM $AlCl_3$. None of the other anions tested reproduced the effect of F^-. When GTPase activity was assayed at 4°C for 1 h, the degree of inhibition by NaF or NaF plus $AlCl_3$ was dependent on prior incubation of transducin subunits with these additions. At 4°C, 3 h were required to achieve maximal inhibition. At 30°C, inhibition was more rapid, reaching a maximum in 20-30 min.

GTPase activity was also inhibited by both decavanadate and monovanadate in a concentration-dependent manner. The extent of mximal inhibition by monovanadate was, however, only half of that by decavanadate, which was, therefore, used for further experiments. Inhibition was rapid, with ∿90% inhibition by 5 mM decavanadate after 5 s at 4 or 30°C. With 2.5 mM NaF, which inhibited GTPase activity ∿20%, inhibition by low concentrations of decavanadate was almost additive. In the presence of 5 mM decavanadate, NaF produced no further inhibition.

Binding and Release of Guanyl Nucleotides: After incubation of transducin subunits and rhodopsin for 30 min at 4°C with NaF or NaF plus $AlCl_3$, Gpp(NH)p binding (at 4°C) was decreased 15% and 35%, respectively. Inhibition was greater, 45% and 80%, respectively, when the incubations were performed at 30°C for 30 min. The effects of NaF and NaF plus $AlCl_3$ on [^{3}H]GTP binding

were essentially the same as those observed with [^{3}H]Gpp(NH)p. After binding of [^{3}H]GTP, the time course of release of label (presumably [^{3}H]GDP) was similar to the time course of [^{3}H]Gpp(NH)p binding. Incubation with NaF or NaF plus $AlCl_3$ for 30 min at 4°C following [^{3}H]GTP binding decreased the subsequent release of [^{3}H]GDP by 20-25%. When incubation with NaF or NaF plus $AlCl_3$ was carried out for 30 min at 30°C inhibition of release was 60% and 85%, respectively.

Like NaF plus $AlCl_3$, decavanadate inhibited [^{3}H]Gpp(NH)p binding to transducin. Inhibition of binding occurred at concentrations of decavanadate that decreased GTP hydrolysis. Following [^{3}H]GTP binding, the release of the radioactivity (presumably [^{3}H]GDP) from transducin was rapid with a half-life of about 1 min. In the presence of 5 mM decanavadate, ∿90% of [^{3}H]GDP remained bound after 20 min. The rate of hydrolysis bound GTP (single turnover rate) was not affected by 5 mM vanadate. Two different methods used to determine the single turnover rate produced essentially the same result. At 4°C, the half-life of bound GTP varied from 2 min to 5 min with different preparations of transducin. At room temperature, the half-life was ∿ 30 s.

T_α Binding to Rhodopsin and ADP-ribosylation by Pertussis Toxin: As reported by Fung, $T_{\beta\gamma}$ was required for significant binding of T_α to rhodopsin. Incubation with NaF or NaF plus $AlCl_3$ inhibited T_α binding (in the presence of $T_{\beta\gamma}$) by 25 and 45%, respectively. Incubation of transducin with decavanadate at 4°C for 30 min inhibited T_α binding in a concentration-dependent manner that correlated well with the inhibition of GTP hydrolysis and GTP binding.

ADP-ribosylation of T_α by pertussis toxin was significantly enhanced in the presence of $T_{\beta\gamma}$; the extent of ADP-ribosylation without $T_{\beta\gamma}$ was 1/10 that with $T_{\beta\gamma}$. In the presence of $T_{\beta\gamma}$, with or without rhodopsin, pertussis toxin-catalyzed ADP-ribosylation of T_α was inhibited 35-40% by NaF and 45-50% by NaF plus $AlCl_3$. Although decavanadate slightly inhibited ADP-ribosylation in the absence of $T_{\beta\gamma}$, with $T_{\beta\gamma}$ much greater inhibition was observed. A similar result was obtained in the absence of rhodopsin.

These studies are consistent with the conclusion that fluoride (plus $AlCl_3$) and vanadate enhance the dissociation of T_α from $T_{\beta\gamma}$. This results in the inhibition of GDP-GTP exchange, and thus GTP hydrolysis. Since it appears that the active substrate for pertussis toxin is $T_\alpha \cdot T_{\beta\gamma}$, dissociation induced by fluoride may also be responsible for the inhibition of ADP-ribosylation. The dissociating effect of NaF (plus $AlCl_3$) on transducin is analogous to its action on G_s and G_i, which can result in adenylate cyclase activation or inhibition. Vanadate probably causes dissociation of G_s and G_i as has been shown for transducin, accounting for its reported effects on cyclase activity.

Significance to Biomedical Research and the Program of the Institute: The hormone-sensitive adenylate cyclase system is critical to the regulation of the cardiopulmonary system. Various inhibitory or stimulatory agonists

affect cells by controlling the rate of cAMP generation; drugs currently in clinical use are directed at modifying the adenylate cyclase activity. Understanding the molecular basis for the interaction of these agents with adenylate cyclase-linked receptors and subsequently of the active receptor complexes with the couplingproteins and catalytic unit of cyclase may further the development and design of therapeutic agents.

Proposed Course: 1) Isolate guanyl nucleotide-binding proteins showing immunological and functional similarities to G_i and G_s.

2) Define the interaction of these guanyl nucleotide-binding proteins with membrane receptors.

Publications: Tsai, S.-C., Adamik, R., Kanaho, Y., Hewlett, E. L., and Moss, J.: Effects of Guanyl Nucleotides and Rhodopsin on ADP-ribosylation of the Inhibitory GTP-binding Component of Adenylate Cyclase by Pertussis Toxin. J. Biol. Chem. 259: 15320-15323, 1984.

Kanaho, Y., Moss, J., and Vaughan, M.: Mechanism of Inhibition of Transducin GTPase Activity by Fluoride and Aluminum. J. Biol. Chem., in press.

Tsai, S.-C., Adamik, R., Moss, J., Vaughan, M., Manne, V., and Kung, H.: Effects of Phospholipids and ADP-Ribosylation on GTP Hydrolysis by E. Coli-Synthesized HA-RAS p21. Proc. Natl. Acad. Sci. USA, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00630-06 CM

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Metabolism of Fatty Acids in Fibroblasts from Patients with Lipid Abnormalities

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Joel Avigan, Ph.D. Research Chemist CM, NHLBI

COOPERATING UNITS *(if any)*

Department of Pediatrics, Medical College of Virginia (Dr. W.B. Rizzo); Department of Pediatrics, University of Umeå, Sweden (Dr. O. Hernell and co-workers).

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.3	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The feasibility of competitive replacement of potentially harmful fatty acids associated with certain neurological diseases with a common fatty acid was successfully tested in vitro and in vivo; in the latter case, by dietary supplementation. The abnormally elevated serum concentration of hexacosansate in adrenoleukodystrophy was substantially decreased following long term feeding with triolein. This phenomenon could be useful in treatment of these patients.

In a continuing work on Δ5 and Δ6 fatty acid reductases in human fibroblasts no decline was observed in these enyzmatic activities with increasing passage number of cultures and cell senescence.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives: 1) To explore means by which potentially harmful fatty acids in certain metabolic diseases, e.g., hexacosanoic acid in adrenoleukodystrophy (ALD), could be reduced by replacement with normally occurring fatty acids. 2) To investigatethe absolute and relative activities of fatty acid desaturases in human skin fibroblasts and detect conditions when these activities are impaired.

Methods Employed: Human skin fibroblasts were grown by conventional methods. Long and very long chain fatty acid were quantified through thin layer and gas chromatography procedures as described earlier. The diet of patients with ALD was supplemented with triolein and the serum fatty acid composition was determined.

Major Findings: 1) The previously reported in vitro effect of oleic acid on hexacosanoic acid levels was shown in a preliminary study to be operating in vivo in two ALD patients who were fed with triolein and whose serum hexacosanoic acid was significantly reduced (from 2.6 to 0.6 µg/ml and from 1.1 to 0.5 µg/ml). The hexacosanoic concentration in an ALD heterozygote declined to normal range following such treatment.

2) In experiments in which $\Delta 5$ and $\Delta 6$ fatty acid desaturases were assayed in fibroblasts cultures of different age it was shown that these activities are not greatly changed with the aging of cultures in the wide range of passage 5 to passage 30. In this in vitro system, the hypothesis of a waning desaturase activity with increasing age could not be therefore confirmed.

Significance to Biomedical Research and the Program of the Institute:
1) Elevation of some rare fatty acids occurs in certain metabolic diseases, such as, of hexanoic acid and in adrenoleukodystrophy, and at phytanic acid in Refsum's Disease. Reduction in tissue concentration of these compounds through dietary intervention may constitute on effective treatment.

2) Enzymes acting in metabolism of linoleic acid and in its conversion to arachidonic acid and to other polyunsaturated compounds are essential to normal cell physiology.

Proposed Course: It is hoped that the studies on the effect of oleate on patients with ALD and Refsum's disease could be continued.

Publications:

Avigan, J. Campbell, B. D., Yost, D. A., Hernell, O., Holmgren, G., and Jagell, S.F.: Sjogren-Larsson Syndrome: Δ^5- and Δ^6-fatty acid desaturases in skin fibroblasts. Neurology 35: 401-403, 1985.

Rizzo, W.B., Watkins, P.A., Phillips, M.W., Cranin, D., Campbell, B., and Avigan, J.: Adrenoleukodystrophy: Oleic Acid lowers Fibroblast Saturated C22-26 Fatty Acids. Neurology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00634-05 CM

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of cGMP-Stimulated Cyclic Nucleotide Phosphodiesterase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Hideo Wada, M.D., Ph.D.	Visiting Fellow	CM, NHLBI
Others:	Vincent C. Manganiello, M.D., Ph.D.	Head, Section on Biochemical Physiology	CM, NHLBI
	Seiko Murashima, M.D., Ph.D.	Visiting Fellow	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS *(if any)*

Molecular Disease Branch, NHLBI (J. C. Osborne, Jr., Ph.D.)

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.3	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Several years ago the cGMP-stimulated cyclic nucleotide phosphodiesterase (PDE) was purified from calf liver. We now report that kinetics of cAMP hydrolysis by this PDE are best described according to the rate equation for a two-site competitive model for allosteric enzymes. According to this model, the PDE exists in two conformations, a "high" and "low" affinity state; binding of substrate (S), effectors (E), or certain competitive inhibitors (I) to the "low affinity" state induces allosteric transitions to the "high affinity" state. This model also accounts for earlier data which indicated that certain I (i.e., IBMX, papaverine, dipyridamole) could apparently mimic S, bind to the low affinity state, induce allosteric transitions to the "high affinity" state and reduce napp, and stimulate hydrolysis of low S. At high S, I competes with S at catalytic sites. Equilibrium binding constants for S and a number of I of the methylxanthine type to high and low affinity states were estimated. Our findings suggest that although a number of derivatives bound to the "low affinity" state as well as S, only two were nearly as effective as S in binding to the "high affinity" state. Thus, structural determinants and requirements are more stringent for binding to the high than to the low affinity state. During allosteric transitions, the topography of specific hydrophobic domains of the enzyme may be altered.

Both temperature and pH alter catalytic activity as well as allosteric transitions. Low temperature (5°C) promotes transitions to the high affinity state, as does incubation at 30°C at high pH (9.5-10) in the presence of MgCl2 MgCl2.

These data indicate that the enzyme possesses independently regulated regulatory and catalytic sites with discrete topographical features and that allosteric transitions involve alterations in affinity for S rather than in Vmax.

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Project Description:

Objectives: To characterize the physical and enzymatic properties and regulation of the cGMP-stimulated cyclic nucleotide phosphodiesterase (EC 3.4.1.17); to define effects of phosphodiesterase inhibitors on the purified enzyme and two other "low K_m" cAMP phosphodiesterases, with the possibility of idenifying specific inhibitors that may be useful for investigating activity of individual phosphodiesterases in intact cells.

Methods Employed: Our purification and characterization of the cGMP-stimulated phosphodiesterase from calf liver supernatant is published (Yamamoto et al., J. Biol. Chem. 257; 12526-12533, 1983.)

Major Findings: The purified cGMP-stimulated PDE hydrolyzes cAMP and cGMP with positively cooperative kinetics. At substrate (S) concentrations below K_m^{app}, hydrolysis of one cyclic nucleotide can be stimulated by the other. cGMP is the preferred S and effector (E).

Earlier data demonstrated that certain inhibitors (I) could mimic S, induce allosteric transitions and reduce n_{app} and stimulate hydrolysis of low S. At S concentrations above K_m^{app}, I competed with S at catalytic sites.

Kinetics of cAMP hydrolysis were analyzed in accord with various models for allosteric enzymes. The data for the cGMP-stimulated PDE were best described by the two-site competitive model for allosteric enzymes. According to this model, the PDE exists in 2 states, a "high affinity" and a "low affinity" state; binding of S, E, or I to the "low affinity" state induces allosteric transitions to the "high affinity" state. Allosteric transitions involve alterations in affinity, not V_{max}. With the model, at high S, with the enzyme in the activated state, I competes with S at catalytic sites. These considerations were consistent with our earlier data which demonstrated that competitive inhibitors did increase hydrolysis of low S and inhibit hydrolysis of high S.

Primary data were analyzed directly as initial velocity versus substrate concentration at multiple different levels of cAMP and I, of the methyl xanthine type, using the general rate equation for the two site competitive model.

$$\frac{v}{V_{max}} = \frac{\frac{[S]^2}{K_S^2} + \frac{[S]}{K_S} + \frac{[S][I]}{B K_S K_I}}{A + \frac{[S]^2}{K_S^2} + \frac{2[S]}{K_S} + \frac{2[S][I]}{BK_S K_I} + \frac{2A[I]}{K_I} + \frac{A[I]^2}{CK_I^2}}$$

where AK_S and K_I are equilibrium dissociation constants for binding of S or I respectively to the ligand-free or low affinity form; and K_S and BK_I are equilibrium binding constants of S and I to the enzyme - S complex or high affinity form.

Initial rates of cAMP hydrolysis in the absence of I (for 12-14 different concentrations of S) from 19 experiments were fit to the rate equation, yielding best fit values of K_S = 2.4 ± .8 μM

AK_S = 410 ± 140 μM

With K_S and AK_S thus fixed, for individual experiments, initial rates of cAMP hydrolysis (12-14 different concentrations) in the presence of various concentrations of I were fit to the rate equation to determine K_I and BK_I. Data sets were analyzed simultaneous using the Marquardt-Levenberg curve-fitting method. Parameter values were adjusted by iteration to minimize the sum of the squares of the deviation between experimental and theoretical data. Values were estimated for K_I and BK_I, equilibrium dissociation constants for I to the "low affinity" and "high affinity" states, respectively. Values for K_I and BK_I were then compared to values for AK_S (410 ± 140 μM) and K_S (2.4 ± .8 μM), equilibrium dissociation constants for cAMP to the "low affinity" and "high affinity" states respectively. Several methylxanthine derivatives especially 1 methyl-3-isobutyl xanthine (IBMX) and 1 methyl-3-isopropyl xanthine bound as effectively or more effectively than cAMP to the low affinity state. Other derivatives with bulky substitutions (i.e., hexyl) in the 1 and 3 position or with 7 methyl substitutions were less effective than cAMP in binding to the low affinity state. From estimates of BK_I, almost none of the derivatives was as effective as cAMP in binding to the high affinity state; only two, IBMX and 1 methyl-3-isopropyl xanthine exhibited BK_I values (1.6 ± .3 and 4.2 ± 1.5 μM, respectively) close to the K_S value for cAMP (2.4 ± .8 μM). These findings suggested that structural determinants and requirements were more stringent for binding to the high affinity state than to the low affinity state and that allosteric transitions may alter topography of specific hydrophobic domains at binding sites.

Taken together with the results of others these findings suggest that cAMP may be oriented in the anti conformation during overall reaction of binding/hydrolysis of cAMP and release of 5' AMP. Such a conformation would orient the purine nucleus to facilitate interaction of substitutions, especially at the 1, 3, and 7 positions with appropriate domains on the enzyme. These domains are apparently such that short hydrocarbon chains in the 1 and 3 position enhance binding to the low affinity state. During allosteric transitions to the high affinity state, the topography of this site is altered so that only short chain substitutions in the 3 position promote binding; substitutions in the 1 position reduce binding to the high affinity sites; bulky substitutions in the 1 and 3 position reduce binding to both high and low affinity states. In the anti conformation, most 8 substituted derivatives would put a considerable strain on the cyclic phosphate ring; most of these derivatives, as might be predicted, are poor substrates and effectors. The importance of increased electron density at the N-7 position of the purine ring is also emphasized with cAMP in the anti conformation. This electron dense nucleus of the purine ring could interact with a positively charged domain on the enzyme during cAMP binding and hydrolysis. Addition of a 7 methyl substitution to several compounds reduces their effectiveness in binding to and inhibiting the PDE relative to original compound, i.e., caffeine (1, 3, 7 trimethylxanthine), 1, 7 dimethylxanthine and 1, 3 dipropyl, 7 methylxanthine are less effective in binding and are less effective inhibitors than theophylline (1, 3 dimethylxanthine); 1, 3 dipropyl,

7 methylxanthine and 1, 3 dipropylxanthine, respectively. On the other hand, 7 (2 chloro)ethyl theophylline is more effective than theophylline. 7-methyl substitutions would decrease electron density and 7 (2 choloro)ethyl would increase electron density at the N-7 position. The former would compete less favorably than the latter for a positively charged enzyme domain.

IBMX seems to be a more effective inhibitor than theophylline for this as well as several other PDE types; this may suggest the existance of a common hydrophobic domain as a general topographic feature of cyclic nucleotide binding sites in these enzymes.

Temperature and pH have been found to affect both catalytic properties and cooperative interactions of the cGMP-stimulated PDE.

Temperature: As temperature of the PDE assay increased from 5°C to 45°C, V_{max} for cAMP and cGMP hydrolysis increased. From a plot of log V_{max} vs $1/T$ the activation energy (Ea) for cAMP and cGMP was estimated; at 30°C, ΔH ($\Delta H = E_a + RT$) was approximately 11,000 Cal/mol for both cAMP and cGMP hydrolysis.

At S concentratins below K_m^{app}, however, initial rates of cAMP hydrolysis were highest at 5°C.

At all assay temperatures studied (5°-45°C) increasing concentrations of cGMP, up to 5-10 μM, stimulated hydrolysis of 0.5 μM [^{3}H]cAMP; higher cGMP concentrations reduced activity. Basal activity was lowest, and fold-stimulation by cGMP highest at 45°C. At several temperatures, apparent activation constants (K_a^{app}) for cGMP stimulation of cAMP hydrolysis were estimated. K_a^{app} was lower at 5°C ($\sim$ .2 μM cGMP) than at 45°C ($\sim$ 3 μM cGMP), indicating an enhanced affinity for effector at 5°C.

Hill coefficients for cAMP and cGMP hydrolysis approached 2 at 45°C and 1 at 5°C; K_m^{app} for cAMP and cGMP was lowest at 5°C. These findings suggest that low temperature induces transitions to the "high affinity" state (reduced napp, increased affinity for substrates and effectors) and also reduces catalytic activity (decreased V_{max}).

We have previously shown that in assays at 30°C certain competitive inhibitors mimic S, induce allosteric transitions to the high affinity state and increase hydrolysis of low S. In the "activated" state, i.e., in assays performed at S concentrations above K_m^{app}, inhibitors compete with S at catalytic sites. In assays at 5°C, IBMX and papaverine did not stimulate, but did inhibit hydrolysis of low S, further suggesting that low temperature induced transition to the "high affinity" or "activated" state. In assays at 45° papaverine and IBMX did stimulate hydrolysis of low S, indicating that at 45°C, as of 30°C, the enzyme is in the "low affinity" state and that inhibitors can induce transition to the "high affinity" states. At 5° or 45°C, IBMX and papaverine inhibited hydrolysis of 50 μM cAMP.

Effects of pH: In the activated state, i.e., at 250 μM [^{3}H]cAMP, 1 or 250 μM [^{3}H]cGMP, or 0.5 μM [^{3}H]cAMP plus 1 μM cGMP, hydrolysis was maximal at pH 7.5-8.0, in assays at different pH (6.0-10.5). Hydrolysis of 0.5 μM [^{3}H]cAMP or 0.01 μM [^{3}H]cGMP, concentrations well below K_m app for each S, was, however, maximal at pH 9.5. Although hydrolysis of 0.5 μM [^{3}H]cAMP increased with pH from 7.5 to 9.5, cGMP stimulation of cAMP hydrolysis decreased. As pH increased or decreased form 7.5, $K_m$app increased, V_{max} and Hill coefficients (N_{app}) decreased. At pH 7.5, N_{app} for cAMP hydrolysis was $\sim$ 1.8; at pH 6.0 or 9.5, $\sim$ 1.0. Thus, assay pH affects both catalytic (V_{max}) and allosteric (N_{app}) properties. Enzyme was therefore incubated (30°, 5 min, 5 mM Mg^{++}) at either pH 7.5 or 9.5 before assay at pH 7.5. Prior exposure to pH 9.5 did not alter V_{max} (assayed at pH 7.5), but did reduce $K_m$app, markedly increased hydrolysis of 0.5 μM [^{3}H]cAMP, and reduced N_{app} to $\sim$ 1.0. Prior incubation at pH 6.0-10.0 did not alter V_{max}, assayed at pH 7.5. After incubation at pH 10.0, in assays at pH 7.5 hydrolysis of 0.5 μM [^{3}H]cAMP was maximally increased and similar in the absence or presence of 1 μM cGMP. These results, which indicate that incubation at high pH (9.5-10) promotes irreversible allosteric transitions (N_{app}) and reversible changes in catalytic activity (V_{max}), are consistent with the presence of catalytic and allosteric sites which can be regulated independently.

Significance to Biomedical Research and the Program of the Institute: Many biochemical effectors, such as hormones and drugs, exert their effects on target cells by altering metabolism of cyclic nucleotides. Understanding the regulatory properties of the phosphodiesterases involved in the degradation of the cyclic nucleotides is important for understanding mechanisms that regulate physiological and pathological processes in mammalian cells.

Proposed Course: Continued characterization of the enzyme with regard to regulatory mechanisms.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00636-04 CM

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Particulate cAMP Phosphodiesterase in 3T3-L1 Fatty Fibroblasts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Martha L. Elks, M.D., Ph.D.	Medical Staff Fellow	CM, NHLBI
Others:	Vincent C. Manganiello, M.D., Ph.D.	Head, Section on Biochemical Physiology	CM, NHLBI
	Marilyn Jackson, Ph.D.	Staff Fellow	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metaboloism	CM, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

3T3-L1 fibroblasts differentiate in culture to develop phenotypic characteristics of adipocytes. Differentiation can be induced by incubation of confluent fibroblasts for 72 hrs with insulin, IBMX and dexamethasone.

Soluble cAMP PDE activity in undifferentiated fibroblasts as well as adipocytes is very sensitive to inhibition by Ro 20-1724, but not cilostamide, whereas adipocyte particulate cAMP PDE is very sensitive to inhibition by cilostamide, but not Ro 20-1724. IBMX readily inhibits both soluble and particulate cAMP PDE activities. Ro 20-1724, but not cilostamide, can substitute for IBMX in inducing differentiation. These and other experiments suggest an important role for soluble PDE in the regulation of differentiation.

In adipocytes, cilostamide enhanced basal and isoproterenol-stimulated lipolysis to a greater extent than Ro 20-1724, but had a lesser effect on cAMP content than Ro 20-1724. The antilipolytic effect of insulin was inhibited by cilostamide but not Ro 20-1724. These findings suggest that particulate cAMP PDE is important in metabolism of cAMP involved in regulation of lipolysis, as well as in the antilipolytic action of insulin.

Incubation of adipocytes with isoproterenol or the antilipolytic agents insulin and PIA for 6-10 min resulted in activation of the particulate cAMP PDE. PIA also inhibited isoproterenol-stimulated adenylate cyclase activity. The effects of insulin and PIA, but not isoproterenol, on PDE activation were inhibited by pertussis toxin, suggesting a role for guanyl nucleotide binding proteins in PDE regulation and/or insulin action. Insulin did not apparently increase hydrolysis of phosphatidyl inositol or activate protein kinase C. Phorbol ester did induce "translocation" of protein kinase C but did not alter insulin's effects on PDE, lipolysis, or glucose transport.

3/1

Project Description:

Objectives: To define mechanisms for control of cAMP phosphodiesterase activity, cAMP content, and lipolysis in 3T3-L1 adipocytes including effects of thyroid hormone, phosphodiesterase inhibitors and other agents.

Methods Employed: 3T3-L1 fibroblasts, obtained from Dr. M. D. Lane of Johns Hopkins University, were grown to confluence in six-well culture plates. Two days later, cells were treated with 100 nM dexamethasone, 1 μM insulin, and 0.1 mM methylisobutylxanthine (IBMX) for 72 hr, then incubated in fresh growth medium. Within a week cells developed characteristics of mature adipocytes. At stages in development of the adipocyte phenotype, cells were exposed to hormones or drugs, harvested by scraping, and homogenized. After centrifugation (100,000 x g, 45 min), samples of supernatant or particulate fractions were assayed for cAMP phosphodiesterase activities. Lipolysis was measured as glycerol production in Hanks' medium with bovine serum albumin, 10 mg/ml. Confluent fibroblasts or differentiated adipocytes were incubated for usually 18 hrs with [^{3}H]-myoinositol. Hydrolysis of ^{3}H-phosphatidyl inositol and accumulation of [^{3}H]-inositol phosphates were monitored by the methods described by Berridge et al. (Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvine, R.F., Biochem. J. 212: 473-482, 1983). Protein kinase C activity was measured in extracts and subcellular fractions from adipocytes incubated with insulin and phorbol esters by modification of procedures desribed by Anderson and co-workers (Kraft, A.S. and Anderson, W.B., Nature 301: 621-623, 1983).

Major Findings: Particulate cAMP phosphodiesterase activity from adipocytes was very sensitive to inhibition by cilostamide in an apparently competitive fashion. On the other hand, supernatant cAMP phosphodiesterase activity was very sensitive to inhibition by Ro 20-1724 in an apparently competitive fashion. IBMX inhibited both supernatant and particulate cAMP phosphodiesterase activities. The antilipolytic effect of insulin was not affected by Ro 20-1724 but was prevented by cilostamide or IBMX. There were no direct effects of these agents on the cAMP-dependent protein kinase nor did they interfere with the effects of insulin on glucose uptake. These findings suggest that much of the antilipolytic effect of insulin may be secondary to the activation of particulate cAMP phosphodiesterase.

Phenyl-isopropyl adenosine, (PIA), a non-hydrolyzable analog of adenosine was used to evaluate the mechanism of antilipolytic effects of adenosine in 3T3-L1 adipocytes. As in other systems, PIA was antilipolytic in these cells and inhibited adenylate cyclase in the presence of GTP. At higher concentrations of isoproterenol, PIA was more effective than insulin in inhibiting lipolysis. At maximally effective concentrations, the two were not additive. In addition to the effects on cyclase, PIA activated particulate cAMP phosphodiesterase, as did insulin. PIA was more effective than NECA (5'-N-ethylcarboxamide-adenosine) or adenosine itself in increasing particulate cAMP PDE; 2'5' dideoxyadenosine (10 μM) was without effect on PDE activity. As with insulin, the effect of PIA on particulate cAMP phosphodiesterase could be blocked by prior treatment of adipocytes with pertussis toxin. The antilipolytic effect

of insulin was not prevented by treatment of the cells with adenosine deaminase which prevented accumulation of adenosine produced by the cell. Thus, the antilipolytic effect of adenosine may be secondary to effects on both adenylate cyclase as well as cAMP phosphodiesterase, while that of insulin may occur via activation of particulate cAMP phosphodiesterase, not mediated by elaboration of adenosine.

Confluent 3T3-L1 fibroblasts and differentiated adipocytes were incubated for 18 hr with [^{3}H] myoinositol and then incubated with insulin (in the presence of LiCl to prevent hydrolysis of inositolphosphate) for 5-30 min. Under these conditions no effect of insulin on hydrolysis of [^{3}H]-phospho inositides or accumulation of [^{3}H]inositol phosphates were observed. Several other agonsits (i.e., bradykinin, vasopressin, Con A, and histamine) were also without effect.

Incubation of adipocytes with phorbol ester resulted in increased protein kinase C activity in adipocyte particulate fractions (8,900 xg, 3 min); incubation of intact adipocytes with insulin was without effect either on "translocation" of protein kinase C activity or on activity recovered in particulate or soluble fractions isolated from adipocyte homogenates. Incubation of intact adipocytes with phorbol ester had no effect on basal or insulin-stimulated hexose transport or particulate cAMP PDE activity or insulin-inhibited lipolysis.

Significance to Biomedical Research and the Program of the Institute: Since cAMP is an important mediator of a number of metabolic processes, it is important to understand those mechanisms involved in the regulation of both production (via adenylate cyclase) and destruction (via cyclic nucleotide phosphodiesterases). In particular (1) Cyclic nucleotide phosphodiesterases comprise a complex group of enzymes. It is not known as to whether the individual members of this group serve specific functions in specific cell types. Out data suggest that in adipocytes, a specific PDE, the particulate cAMP PDE, is important in the metabolism of cAMP involved in regulation of lipolysis. Activation of this specific PDE may be important in the antilipolytic actions of insulin and adenosine. (2) This work suggests that guanyl nucleotide binding proteins are involved in the regulation of cAMP metabolism as well as synthesis. (3) Heretofor, the antilipolytic action of adenosine has been thought to occur via adenosine-inhibition of adenylate cyclase. This work suggests that in 3T3-L1 adipocytes adenosine inhibits lipolysis via concerted efforts on both adenylate cyclase and the particulate cAMP PDE. (4) This work also suggests that the "permissive" effects of both glucocorticoids and thyroid hormone on hormone-stimulated cAMP accumulation and lipolysis also occur in part via concerted alterations in both adenylate cyclase and PDE systems.

Proposed Course: Further investigation of (1) the mechanism of activation particulate cAMP PDE by insulin and adenosine

(2) the role of this PDE in the antilipolytic action of insulin

(3) the role of guanyl nucleotide binding proteins in the regulation of PDE activity and/or insulin action

(4) the mechanisms involved in the differentiation of the particulate cAMP PDE.

Publications:

Elks, M. L., Manganiello, V. C., and Vaughan, M.: Effect of Dexamethasone on Adenosine 3', 5'-Monophosphate Content and Phosphodiesterase Activities in 3T3-L1 Adipocytes. Endocrinology 115: 1350-1356, 1984.

Elks, M. L. and Manganiello, V. C.: Selective Effects of Phosphodiesterase Inhibitors on Different Phosphodiesterases, Adenosine 3', 5'-Monophosphate Metabolism, and Lipolysis in 3T3-L1 Adipocytes. Endocrinology 115: 1262-1268, 1984.

Elks, M. L. and Manganiello, V. C.: Antilipolytic Action of Insulin: Role of cAMP Phosphodiesterase Activation. Endocrinology 116: 2119-2121, 1985.

Elks, M. L. and Manganiello, V. C.: A Role for Soluble cAMP Phosphodiesterase in Differentiation of 3T3-L1 Adipocytes. J. Cell. Physiol. 124: 1985, in press.

Elks, M. L. and Manganiello, V. C.: Effects of Thyroid Hormone on Regulation of Lipolysis and cAMP Metabolism in 3T3-L1 Adipocytes. Endocrinology, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00638-03 CM

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transducin GTPase: Genes for GTP-Binding Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Joel Moss, M.D., Ph.D.	Head, Section on Molecular Mechanisms	CM, NHLBI
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	Maureen Hoffman-McEnery, Ph.D.	Staff Fellow	CM, NHLBI
	Suzanne Czarnecki, Ph.D.	Staff Fellow	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS *(if any)*

Division of Biochemistry and Biophysics, Office of Biologics, Center for Drugs and Biologics, FDA, Bethesda, Maryland (Dr. Darrell Liu); Laboratory of Molecular Hematology, NHLBI, NIH, Bethesda, Maryland (M.D. Schafer).

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Guanine nucleotide-binding proteins (GNP) are critical to the regulation of cellular metabolism. Three GNP's, Gs, Gi, and transducin, composed of α, β, and γ subunits mediate hormonal stimulation of adenylate cyclase, hormonal inhibition of adenylate cyclase, and light activation of phosphodiesterase of retinal rod outer segments, respectively. A fourth GNP, Go, interacts with inhibitory hormone receptors, but does not appear to affect cyclase activity. A fifth GNP, p21, is a product of a proto-oncogene involved in the regulation of cell growth. To define the molecular mechanisms by which these GNPs couple external stimuli to intracellular events monoclonal and polyclonal antibodies were prepared and cDNA clones were isolated for some of these proteins. Three monoclonal antibodies prepared to purified transducin (T) reacted with Tα but not Tβγ and inhibited the ability of Tα in the presence of rhodopsin and Tβγ to hydrolyze GTP. Another monoclonal antibody against Tα cross-reacted with Giα, Goα and p21. All 4 antibodies reacted with the amino-terminal region of transducin. These studies are consistent with the presence of common epitopes in GNPs. Immunization of rabbits with Tβγ resulted in the production of anti-idiotypic antibodies against rhodopsin, consistent with the hypothesis that Tβγ binds to rhodopsin.

To define further the regulation of transducin, a cDNA clone for the γ subunit was isolated from a λgt10 cDNA library. Using the cDNA clone a single size class of message was demonstrated in total retinal RNA by Northern blotting; no homologous messages were detected in either brain or liver RNA. Polyclonal antibodies to Tγ did not cross-react with γ proteins from brain or liver. Thus, γ subunits appear to be tissue-specific.

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Project Description:

Objectives: To clone the genes for the subunits of transducin and to characterize the immunological properties of the guanyl nucleotide-binding proteins. Coupling of surface receptors to intracellular target enzymes is mediated through guanyl-nucleotide binding proteins, which include the G proteins of the hormone-sensitive adenylate cyclase system, and transducin of the light-sensitive phosphodiesterase system. The hormone-sensitive adenylate cyclase and light-sensitive phosphodiesterase systems are composed of at least three types of proteins. These include receptors, for stimulatory and inhibitory agonists in the cyclase system, and the photon receptor rhodopsin in the phosphodiesterase system, the catalytic unit, which in the case of cyclase, is responsible for cAMP formation, and in the visual system, for cGMP degradation, and the GTP-binding proteins G_s, G_i, and transducin, which couple the receptor to the catalytic unit.

The guanyl-nucleotide binding proteins are heterotrimers of α, β, and γ subunits. The α subunits are similar in size (39-45 kDa), have a high affinity GTP-binding site, and possess GTPase activity. The β subunits (35 kDa) are highly conserved structurally and are required for maximal GTPase activity. The γ subunit is bound to the β subunit; its function is not known. In addition to their structural homology, $G_{i\alpha}$ and $G_{\beta\gamma}$ are functionally analogous to T_α and $T_{\beta\gamma}$, respectively, in their interaction with receptors. Activated receptors stimulate the GTPase activity of the α subunit in a reaction enhanced by the $\beta\gamma$ subunits. The α and β subunits of the G proteins and transducin display striking homologies. To better understand the molecular basis by which these proteins regulate metabolic processes, we have begun to clone the genes coding for these proteins, and produce monoclonal and polyclonal antibodies to their subunits. Knowledge of the structure of the genes and the immunological properties of the proteins will provide information concerning the structure and regulation of these proteins and will facilitate studies aimed at understanding the function of these proteins.

Methods Employed: Transducin was prepared as previously described (Watkins, P.A., Moss, J., Burns, D.L., Hewlett, E.L., and Vaughan, M., J. Biol. Chem. 259: 1378-1381, 1984). The α and $\beta\gamma$ subunits were separated using Blue Sepharose CL-6B (Shinozawa, T., Uchida, S., Martin, E., Cafiso, D., Hubbell, W., and Bitensky, M., Proc. Natl. Acad. Sci. USA 77: 1408-1411, 1980). Rhodopsin was purified by published procedures. $G_{i\alpha}$, $G_{s\alpha}$, and $G_{\beta\gamma}$ were purified by procedures developed by Gilman and coworkers. Rabbits were immunized with approximately 500 µg of transducin or transducin α and $\beta\gamma$ subunits emulsified in complete Freund's adjuvant by subcutaneous injection at multiple sites. Booster injections (100-200 µg of protein in incomplete Freund's adjuvant) were administered at intervals of three to five weeks; serum samples were obtained 7-10 days after each injection. Antisera (titer > 1/1000) reacted with all subunits. Monoclonal antibodies were produced using Balb/c mice and SP 2/0 myeloma cells.

The IgG fractions from anti-transducin, anti-$T_{\beta\gamma}$ and anti-rhodopsin serum were obtained by repetitive chromatography of serum on protein A-Sepharose.

The IgG fraction was eluted at pH 2.5. Anti-transducin and anti-rhodopsin activities contained in the same IgG fraction were separated using a transducin-Sepharose prepared by coupling purified transducin to CNBr-activated Sepharose 4B. Purified IgG was applied to the column at pH 8.5, and anti-transducin antibodies were eluted from the column using a buffer of pH 4.5.

Antibodies were detected by using an enzyme-linked immunosorbent assay on nitrocellulose paper. Briefly, transducin, its subunits, or rhodopsin were applied to the paper, which was incubated with rabbit antiserum or purified IgG and then with goat anti-rabbit IgG coupled to horseradish peroxidase. After adding the peroxidase substrate, the colored product was quantified by densitometry to determine amount of antibody reacted with transducin or rhodopsin. GTPase activity was assayed in a system containing rhodopsin reconstituted into phosphatidylcholine vesicles and transducin or its α and $\beta\gamma$ subunits. Rhodopsin was incubated with anti-rhodopsin antibodies for 10 minutes at 4°C before addition of other components and assay of GTPase activity. Southern, Northern and Western blotting was performed and RNA and DNA were isolated by published methods. Oligonucleotide probes were used to identify clones in a λgt10 library.

Major Findings: 1) Cross-reactivity of monoclonal antibodies to transducin. Monoclonal antibodies against transducin subunits were prepared using Balb/c mice and SP2/0 myeloma cells. Four antibodies against T_α were screened for cross-reactivity to α subunits of other G proteins. One antibody recognized $G_{i\alpha}$, $G_{o\alpha}$ and p21, the product of the ras protooncogene. Tryptic peptide mapping established that each monoclonal antibody recognized a 23 kDa fragment derived from the N-terminal region of T_α. The three monoclonal antibodies that recognize only T_α inhibited the GTPase activity of transducin reconstituted with rhodopsin in phosphatidylcholine vesicles.

2) $T_{\beta\gamma}$ interacts with rhodopsin. Two models have been proposed for the interaction of T_α and $T_{\beta\gamma}$ with agonist-receptor complex. In model I, the agonist-receptor complex interacts only with the α subunit; the $\beta\gamma$ subunits stimulate GTPase activity by interacting with α, but not with the receptor. In model II, the agonist-receptor complex interacts with the $\beta\gamma$ subunits as well as with the α subunit. In an attempt to distinguish between these two models, we have employed an immunological approach. It is known that anti-idiotypic antibodies can share conformational similarity with the original antigen. Thus, anti-idiotypic antibodies generated upon immunization with transducin could resemble transducin, specifically, for our purpose, the site of transducin interaction with rhodopsin. If this were the case, these anti-idiotypic antibodies would bind to rhodopsin. The production of anti-rhodopsin antibodies by rabbits immunized with transducin or $T_{\beta\gamma}$ would provide evidence that $T_{\beta\gamma}$ interacts directly with rhodopsin.

Serum from rabbits immunized with transducin or $T_{\beta\gamma}$ reacted with rhodopsin as well as with the immunizing antigen. The rhodopsin- and transducin-binding activity of the purified IgG fractions correlated well with that in whole serum. The reaction of anti-transducin IgG with rhodopsin was approximately 30% of its reaction with transducin. The reaction of anti-$T_{\beta\gamma}$ IgG with rhodopsin was about 150% of the reaction with $T_{\beta\gamma}$. The purified IgG fractions were

fractionated on transducin-Sepharose. The material that did not bind was enriched in rhodopsin-binding activity relative to transducin or $T_{\beta\gamma}$ binding activity. Elution at low pH yielded the expected anti-transducin or anti-T_{β} antibodies. Rhodopsin-binding activity of the eluted material was less than 10% of the total recovered rhodopsin-binding activity.

The rhodopsin-binding antibodies separated from anti-transducin or anti-$T_{\beta\gamma}$ were tested for their effect on rhodopsin-stimulated GTPase. The IgG fractions were incubated with purified rhodopsin for 10 minutes at 4°C. Fractions from both anti-transducin and anti-$T_{\beta\gamma}$ serum inhibited rhodopsin-stimulated GTPase. Activity in the presence of anti-rhodopsin IgG was 40-60% of control values. Because these antibodies appeared after immunization with transducin or $T_{\beta\gamma}$, they are most likely anti-idiotypic antibodies directed against idiotypic anti-transducin or anti-$T_{\beta\gamma}$ antibodies. The generation of these anti-idiotypic antibodies is evidence that $T_{\beta\gamma}$ interacts with rhodopsin directly.

Functional and structural studies have demonstrated that the $\beta\gamma$ complex of transducin appears to serve similar role in the adenylate cyclase and retinal photoreceptor systems. Because $G_{\beta\gamma}$ can interact with rhodopsin and T_{α} to stimulate GTP hydrolysis, it is likely that the site of $G_{\beta\gamma}$ binding to inhibitory receptors will be very similar to the $T_{\beta\gamma}$ binding site on rhodopsin. The anti-idiotypic antibodies that we have reported here should be useful in characterizing the interaction between the $\beta\gamma$ complex and receptor in both systems.

3) Isolation of several cDNAs that code for the γ-subunit of transducin. Screening of a bovine retina cDNA library with 2 sets of oligonucleotides identified several cDNAs which when sequenced contained the entire γ-subunit coding region. Subfragments of these cDNAs have been used in Northern blots of total retinal RNA to determine the size of the reactive endogenous RNA ($\sim$ 500 bases) which is consistent with one size class of T_{γ} messages in retina. Similar Northern blots of brain RNA did not reveal the presence of transducin γ RNA, consistent with the hypothesis that the production of transducin γ is tissue-specific. To further confirm this proposal, total RNA from liver, retina, and bovine brain was translated in a rabbit reticulocyte lysate system. The radiolabeled protein product was immunoprecipitated with antiserum to transducin, followed by addition of protein A-Sepharose. Transducin γ was detected in the *in vitro* translation products of retinal RNA but not brain or liver RNA.

Significance to Biomedical Research and the Program of the Institute: The hormone-sensitive adenylate cyclase plays a critical role in many tissues including the heart and lung by mediating the actions of a number of agents. The hormone response is mediated through proteins that require guanyl nucleotides for activity. These studies will aid in the understanding of the structures, regulation, and functions of these GTP-binding proteins.

Proposed Course: 1) To characterize the clone for the γ subunit of transducin and isolate the gene from a bovine library.

2) To define the specificity of the monoclonal antibodies directed to the subunits of transducin.

3) To identify the epitope in rhodopsin recognized by the anti-rhodopsin antibodies produced by immunization with transducin.

Publications:

Moss, J., Burns, D.L., Hsia, J.A., Hewlett, E.L., Guerrant, R.L., and Vaughan, M.: Cyclic Nucleotides: Mediators of Bacterial Toxin Action in Disease. Annals of Internal Medicine 101: No. 5, 1984.

Vaughan, M. and Moss, J.: Altered regulation of adenylate cyclase after toxin-catalyzed ADP-ribosylation. In Molecular Basis of Cellular Regulation (Current Topics in Cellular Regulation). New York, Academic Press, in press.

Chang, P.P., Halpern, J., Sohn, R.L., Kanaho, Y., Moss, J., and Vaughan, M.: Coupling of Receptors to Signal Transducing Components: Use of Anti-idiotypic Antibodies to Map the Specific Sites of Interaction of Receptors and Guanyl Nucleotide-Binding Proteins. Transactions of Assoc. Am. Physicians, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00639-02 CM

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of a Bovine Rod Outer Segment cGMP Phosphodiesterase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Vincent C. Manganiello, M.D., Ph.D.	Head, Section on Biochemical Physiology	CM, NHLBI
Others:	Joel Moss, M.D., Ph.D.	Head, Section on Molecular Mechanisms	CM, NHLBI
	Martha Vaughan, M.D.	Chief, Laboratory of Cellular Metabolism	CM, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A cGMP phosphodiesterase was extracted from bovine rod outer segments by suspension of rod outer segments in hypotonic medium and further purified by chromatography on AcA 34. Enzyme activity was markedly increased by trypsin; activity was also increased severalfold in the presence of NAD and a mono-ADP-ribosyltransferase from turkey erythrocytes. Activation by trypsin resulted in an increase in Vmax and decrease in Km for cGMP; ADP-ribosylation was accompanied by an increase in Vmax. Activation by trypsin is thought to be related to destruction of a small heat stable inhibitory subunit associated with the holoenzyme. This subunit was prepared by chromatography on Sephadex G-50 of a purified, heat-treated ROS cGMP PDE preparation. Incubation of this inhibitor, NAD, and purified transferase reduced the capacity of this material to inhibit trypsin-activated ROS cGMP PDE; incubation with [^{32}P]NAD and transferase resulted in [^{32}P]ADP-ribosylation of the inhibitor. These findings are consistent with ADP-ribosylation of the inhibitory subunit resulting in increased activity of the ROS cGMP PDE.

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Project Description:

Objectives: To characterize regulatory properties of the light-activated cGMP phosphodiesterase (PDE) from bovine rod outer segments (ROS) and effects of limited proteolysis and covalent modification on activity.

Methods Employed: Purification of enzyme. Hypotonic extracts from ROS were concentrated and chromatographed on AcA 34 columns equilibrated and eluted with 50 mM HEPES, pH 7.4/0.4 M NaCl/1.0 mM EGTA/1 mM NaN_3 plus protease inhibitors leupeptin, pepstatin, and phenylmethylsulfonyl fluoride. Purity was assessed by polyacrylamide slab gel electrophoresis in SDS.

Major Findings: A cGMP PDE was partially purified from hypotonic extracts of bovine retina rod outer segments by chromatography on AcA 34. Polyacrylamide gel electrophoresis under denaturing conditions demonstrated the presence of 3 subunits of $\sim$ 88 and 86 kDa and a low molecular weight peptide with Mr $<$ 14 kDa. This small, heat-stable subunit has been reported to exert inhibitory constraints on ROS cGMP PDE; trypsin-induced activation is thought to be related to destruction of this inhibitory subunit. The heat stable peptide was separated from purified ROS cGMP PDE. cGMP PDE was heated (90°C, 5 min) and centrifuged (100,000 xg, 60'); the supernatant was applied and eluted from a Sephadex G-50 column. Inhibitory activity was monitored by capacity of eluates to reduce activity of trypsin-activated cGMP PDE.

Limited proteolysis of ROS cGMP PDE by trypsin resulted in a marked increase in cGMP PDE activity; activation was accompanied by an increase in V_{max} and slight decrease in Km for cGMP. Incubation of ROS cGMP PDE with a purified turkey erythrocyte ADP-ribosyl transferase (transferase) also resulted in a 2-4 increase in cGMP PDE activity, much less than that observed with trypsin. The activation by transferase required NAD and depended on transferase concentration. Activation was accompanied by an increase in V_{max}, with no change in Km for cGMP. After incubation with transferase, activity was further increased by exposure to trypsin. Maximal activation of the ADP-ribosylated PDE was produced by a lower trypsin concentration than the control PDE. Activation by limited proteolysis rendered the ROS PDE more susceptable to thermal inactivation during incubation at 40°C prior to assay at 30°C. After activation by either limited proteolysis or the ADP-ribosylation the enzyme was less susceptible to inhibition by agmatine. Effects of histone were complex and somewhat different for the enzyme activated by proteolysis or ADP-ribosylation. Lower concentrations of histone ($\sim$ 1-3 µg/ml) inhibited control PDE or activity after limited proteolysis with little or no effect on the ADP-ribosylated enzyme activity. Higher concentrations of histone (3-10 µg/ml) stimulated control or ADP ribosylated activity but did not further activate the trypsin-treated enzyme.

Incubation of the holoenzyme with [^{32}P]-NAD resulted in incorporation of [^{32}P] ADP-ribose into both large and small subunits. Incubation of the purified heat stable inhibitory subunit with NAD and the transferase reduced

the capacity of the subunit to inhibit trypsin-activated cGMP PDE; incubation of [^{32}P]-NAD and the transferase resulted in incorporation of [^{32}P] ADP-ribose into the small peptide. Treatment of the trypsin-activated enzyme with transferase and NAD did not alter the effect of the inhibitory subunit on PDE activity. These data are consistent with an affect of covalent modification (ADP-ribosylation) on the low molecular weight (inhibitor) subunit, resulting in activation of the ROS cGMP PDE.

Significance to Biomedical Research and the Program of the Institute: This phosphodiesterase, which plays a major role in the process of visual excitation, is controlled through a recptor/GTP-binding protein system analogous to that of the hormone-sensitive adenylate cyclase. Thus, information about this enzyme may aid in our understanding the cyclase system as well as the properties of other cyclic nucleotide phosphodieterases, especially the hormone-sensitive particulate phophodiesterase in adipose tissue.

Proposed Course: Characterization of this enzyme with emphasis on reconstitution studis with purified regulatory components of the light-sensitive phosphodiesterase system as well as the hormone-sensitive phosphodiesterase and adenylate cyclase systems.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00641-01 CM

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on Muscarinic Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Joel Avigan, Ph.D. Research Chemist CM, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Cellular Metabolism

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.7	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Extraction and chromatography of muscarinic receptors derived from pig atrial cell membranes yielded an active preparation that was capable of interacting with other components of the receptor-effector complex.

Binding of muscarinic antagonists to human skin fibroblasts was investigated and declining dissociation of the bound ligand in the course of subsequent incubation of 37° was demonstrated. Serum proteins were found to competitively inhibit binding of ligands to whole cells and cell membranes.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives: 1) To prepare a soluble extract of muscarinic receptors of adequate purity for studies of regulation of binding and of modulation of receptor activity by other compounds of the "recognition-activation" complex.

2) To study binding of muscarinic agonists and antagonists to human skin fibroblasts.

Methods Employed: Pig atria, or other tissues were processed through steps that included homogenization, fractional centrifugation and particle washing to obtain muscarinic receptor-rich cell membranes that were subsequently extracted with detergent-containing medium. The extract was chromatographed on a wheat germ agglutinin agarose column from which a receptor-enriched fraction was eluted. Some of the above proceedures were adopted from those of G.L. Peterson, et al. (Proc. Natl. Acad. Sci. USA 81; 4993, 1984). Human skin fibroblasts were grown by conventional methods. Assays of binding of muscarinic ligands were carried out by incubation with labeled antagonists followed by cell washing, in the case of fibroblasts cultures, or rapid filtration and washing in experiments with membrane preparations, or gel filtration (Sephadex) for soluble extracts.

Major Findings: 1) Preparation of muscarinic receptors: Pig atria were found to be more advantageous than bovine brain and atria for the preparation of muscarinic receptors. Work with a large batch in facilities available in the pilot plant of NIA did not prove to be more efficient than processing of small batches on a laboratory scale. One step chromatography of the extract on a lectin-agarose column yielded a fraction with activity that was several-fold higher than the original extract. In a preliminary experiment there was no evidence for substantial contamination with G_i protein based on the apparent absence of ADP-ribosylation by pertussis toxin. Gpp(NH)p (a non hydrolysable analogue of GTP) did not consistently affect binding of ^{3}H-QNB by the partially purified receptor preparation, but did so in the presence of added bovine transducin. The latter acted, therefore, similarly to the G_i regulatory protein in the native complex.

2) Infant-derived human skin fibroblasts display high- and low affinity binding of labeled muscarinic antagonists. Changes subsequent to binding were studied by measuring its reversibility during a post-incubation with an unlabeled agonist or antagonist. Whereas binding of labeled QNB by isolated fibroblast membranes could be competitively reduced by unlabeled ligand, reversibility in labeled whole cells rapidly declined on incubation at 37°. The nature of such changes is still uncertain. Whole human or animal serum as well as several individual serum proteins inhibited competitively the binding of muscarinic ligands to whole cells and cell membranes.

Significance to Biomedical Research and the Program of the Institute: 1) Transmission of a signal by acetyl choline or its hormonal analogues through cell membrane depends on the integrity of the specialized receptor-effector/ complex; studies of conditions at which the complex is functional may contribute to the knowledge of the process.

2) Cultured cells provide a useful system for the study of long term changes in binding of muscarinic ligands resulting from desensitization, endocytosis or other metabolic processes.

Proposed Course: It is proposed to investigate interactions between components of the muscarinic sensitive complex. Changes with time of the ligand-cell bond that follow initial binding will be further studied.

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF CHEMICAL PHARMACOLOGY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1984 through September 30, 1985

A long-term objective of this Laboratory has been the development of several approaches for discovering drugs and other foreign compounds that cause tissue lesions through the formation of chemically unstable metabolites. Although the Laboratory has continued in these efforts during the past year, it has shifted its emphasis toward studies of possible mechanisms by which drugs and their metabolites may exert their toxic effects. Tissues were selected for study primarily because of the vulnerability to toxic metabolites. However, the mast cell, because of its role in inflammatory/allergic reactions, has also been a subject of interest in this Laboratory.

Mechanisms of Toxicity

Halothane. Halothane may be converted to chemically reactive metabolites either by reductive cleavage of a carbon halogen bond to form a radical or by hydroxylation of the carbon hydrogen bond followed spontaneously by dehydrohalogenation to form a trifluoroacetyl halide. Although an acute hepatotoxicity has been associated with the reductive pathway there is evidence that the fulminant type of halothane hepatotoxicity observed in humans may be due to an immune reaction. In accord with this view sera of patients who have recovered from halothane induced hepatoxicity initiate a lymphocyte mediated destruction of hepatocytes from rabbits treated with halothane. We reasoned, however, that if a metabolite of halothane were to serve as a hapten in humans, the adduct formed between the chemically reactive metabolite and a protein should be associated with the external surface of plasma membranes of hepatocytes. Last year, we reported the development of an antibody against the trifluoroacetyl group of halothane. With this antibody we demonstrated that the trifluoroacetyl group was covalently bound to proteins predominantly in the centrilobular region of liver of rats receiving halothane and that some of the covalently bound metabolite was on the surface of hepatocytes. During the past year, we have found that the trifluoroacetyl group forms adducts predominantly with two microsomal proteins having molecular weights of about 54 kD and 59 kD. The 54 kD protein has been identified as an isozyme of cytochrome P-450. The specificity of the covalent binding of the trifluoroacetyl group thus suggests that the reactive metabolite is too short-lived to diffuse from the endoplasmic reticulum to the cell surface. Instead the externally bound adduct appears to be due both to endoplasmic reticulum from dead cells that contaminate the preparations and to cytochrome P-450 within the plasma membrane. These findings raise the possibility that fulminant type of hepatotoxicity might be autocatalytic in that the endoplasmic reticulum released from dead cells may be adsorbed onto neighboring cells and thereby serve as focal points for immune reaction. This possibility is being explored.

Studies with human microsomes from liver biopsy samples obtained about 4 hr after halothane anesthesia revealed that the trifluoroacetyl group may become covalently bound to as many as three proteins having molecular weights similar to those of the cytochrome P-450.

Mechanisms of heme destruction. The administration of carbon tetrachloride and other substances that promote lipid peroxidation in cells results in the destruction of cytochrome P-450 by causing the destruction of heme. Last year we reported that much of the decomposed heme became covalently bound to protein in liver microsomes. During the past year, we discovered that some of the heme decomposition products become covalently bound to the proteins of cytochrome P-450-54kD and cytochrome P-450-52kD. Whether the covalent binding occurs at the active sites of the cytochrome, however, remains to be investigated.

This kind of mechanism of suicide enzyme inhibition may occur with other toxicants. Preliminary experiments suggest that allyl isopropylacetamide, a porphyrinogen, may also inactivate cytochrome P-450 not only by forming adducts with heme, but also by causing covalent binding of heme to the proteins of cytochrome P-450. Whether the cytochrome P-450 suicide inhibitors for specific isozymes of cytochrome P-450 remains to be investigated.

Tunicamycin. The administration of tunicamycin, an antiviral drug, to rats decreases the concentrations of cytochrome P-450 and to a lesser extent NADPH cytochrome P-450 reductase in liver microsomes. The effects, however, are not manifested until 24 hours after the administration of the drug. The drug evokes similar effects in hepatocyte cultures. Although it has been thought that the drug exerts its therapeutic action by blocking glycosylation, we have found that it also decreases protein synthesis as measured by methionine incorporation. The drug apparently acts by decreasing the amount of m-RNA, because it also decreases the translation of mRNA in a rabbit reticulocyte lysate system.

Clofibric acid. The administration of clofibrate to rats promote the proliferation of peroxisomes in liver. During the past year, we demonstrated that both clofibric acid and ciprofibrate cause similar effects in hepatocyte cultures; indeed ciprofibrate was about 6 times as effective as clofibric acid. The drugs apparently act by increasing the amounts of the mRNA's that code for peroxisomal proteins. The induction was inhibited by cycloheximide. Moreover, methionine incorporation into cellular protein and the translation of RNA isolated from cells treated with the drugs were increased. Furthermore, the increases of methionine incorporation in intact cells and in rabbit reticulocyte lysate systems was preferentially greater in catalase and carnitine acetyltransferase, which are markers for the peroxisomes.

Cardiotoxicity caused by analogues of adriamycin. It has long been thought that these drugs cause their toxic effects in heart by increasing the formation of superoxide and hydrogen peroxide and thereby promoting lipid peroxidation. Our studies with cardiomyocytes during the past few years, however, are inconsistent with this mechanism. Hydrogen peroxide generated in cells is preferentially inactivated by glutathione peroxidase to form oxidized glutathione, which was actively excreted from cells. Although adriamycin and daunomycin increase the release of glutathione from the cells, nearly all of the released glutathione was not in its reduced form and not its oxidized form. Moreover, bis-chloroethyl nitrosourea, which is presumably a selective inhibitor of glutathione reductase, did not result in an increase in the release of oxidized glutathione even though the inhibitor potentiates the toxicity of daunomycin.

Furthermore, 4-deoxyadriamycin and the calcium ionophore, A-23187, which do not cause increases in the formation of hydrogen peroxide, nevertheless promoted the release of glutathione without altering the amount of oxidized glutathione within the cells.

The mechanism of toxicity of adriamycin and daunomycin may involve either active transport systems or the permeability of the cardiomyocytes. Both adriamycin and daunomycin decreased the uptake of deoxyglucose, various amino acids and adenine into cardiomyocytes. The decrease in uptake of adenine resulted in a decrease in its incorporation into ATP pools; this decrease may account in part for the rapid decline in the synthesis of ATP within hepatocytes and the decline in ATP pools within the cardiomyocytes that preceed the leakage of lactate dehydrogenase out of the cells.

Mechanisms of Mast Cell Activation and Degranulation

The abundance of mast cells in the small blood vessels, heart and airways makes these sites particularly vulnerable to action of IgE-directed antigens. Apart from its clinical importance the mast cell/basophil system has also become a useful experimental model to study stimulus-secretion coupling mechanisms. For these and other reasons the emphasis of our work has shifted from the study of mechanism of histamine synthesis to that of histamine secretion.

Three years ago we initiated studies in collaboration with James Metcalfe at the University of Cambridge to utilize new techniques to measure cytosolic calcium levels (with quin 2) as well as membrane phosphatidylinositide turnover in mast cells. For convenience our initial studies were conducted with the histamine-releasing RBL clone, the 2H3 cell. These studies demonstrated that increases of cytosolic calcium levels ($[Ca^{2+}]$) were obligatory for histamine release and that extensive hydrolysis of membrane phosphatidylinositides was associated with antigen stimulation. Moreover, the hydrolysis was related directly to receptor aggregation and was not a consequence of Ca^{2+} influx. Increases in ($[Ca^{2+}]$), in quin 2 loaded cells at least, was dependent exclusively on Ca^{2+} influx rather than recruitment of Ca^{2+} ions from internal membrane stores. The data did not distinguish between two possibilities, namely whether Ca^{2+} influx and phosphatidylinositide hydrolysis were independent simultaneous events or whether Ca^{2+} influx was a consequence of phosphatidylinositide hydrolysis. It was clear, however, that increases in ($[Ca^{2+}]$) (henceforth referred to as the calcium signal) and hydrolysis were ATP-dependent steps as neither event occurred in ATP depleted cells. At the NIH we have extended the studies in three ways. We have conducted, 1) experiments with Dr. Henry Metzger with covalently cross-linked oligomers of IgE to obtain precise quantitation of receptor aggregation and signal generation in 2H3 cells, 2) a critical evaluation of the ATP-dependency by careful titration of cellular ATP levels with antimycin-A and 3) a detailed investigation of nonresponsive and partially responsive clones of RBL cells with the expectation that specific defects could be found in the mechanisms that result in hydrolysis of phosphatidylinositides, increased kinase C activity and increases in intracellular calcium.

The oligomers studied included monomers, dimers, trimers and higher oligomers of a monoclonal IgE. The monomers were inactive, but the others induced phosphatidylinositide breakdown, a calcium signal and histamine secre-

tion. Higher oligomers were more active than trimers, and trimers more active than dimers. Irrespective of the type of oligomer tested, the calcium signal was highly correlated with phosphatidylinositide breakdown. From binding studies, it was apparent that occupancy of < 0.1% of the IgE receptors by trimer or higher oligomer was sufficient to generate measurable signals and induce secretion of histamine. In fact, increases in cytosol calcium concentrations were apparent when as few as 50 receptors/cell were occupied. Maximal secretion of histamine occurred with aggregation of < 20% of the receptors whereas the maximal calcium signal or maximal rates of phosphatidylinositide breakdown occurred only with saturating concentrations of oligomer. Signal generation was thus related to receptor occupancy: the cells also appeared to possess more receptors and capacity for phosphatidyl inositide breakdown than was required for the maximum secretory response.

From the above data, aggregation of 3 or more receptors appeared to be a more effective stimulus for signal generation than did aggregation of just 2 receptors. A novel finding was that both signal generation, and histamine secretion, were enhanced further by D_2O (5-30%). D_2O, which was thought to promote histamine release through its action on microtubules, may act instead indirectly through alteration of critical properties of plasma membranes. It did not enhance the activity of the key enzyme (phospholipase C) involved in phosphatidylinositide hydrolysis. Of possible relevance was the recent observation of Metzger and colleagues that common organic solvents in nontoxic concentrations (0.1 to 0.2%) quench phosphatidylinositide breakdown and secretion of histamine in stimulated 2H3 cells. Signal generation was found by us to be sensitive to small temperature changes. For example, increasing the temperature from 37 to 40° resulted in complete suppression of phosphatidylinositide hydrolysis, calcium signal and secretion of histamine. Responses were restored upon decreasing the temperature to 37°. Hence receptor aggregation and stimulation of phosphatidylinositide hydrolysis are probably loosely coupled events in the sense that the system is sensitive to subtle changes that may perturb membrane properties.

Our current studies showed also that signal generation was highly dependent on cellular ATP levels (correlation coefficients were > 0.98) and that it may be subject to feedback modulation through kinase C activation. An activator of this enzyme, the phorbol ester, TPA, in nM concentrations in stimulated cells suppressed both the breakdown of phosphatidylinositides and the increase in calcium signal without affecting histamine secretion. On the other hand activation of kinase C sensitized the cell to calcium signals. TPA markedly potentiated secretion of histamine induced by the calcium ionophore A 23187. However, activation of kinase C (i.e. with TPA) or modest increases in cytosol calcium (i.e. with 25-100 nM A23187) alone did not cause secretion. Both the Ca^{2+} and kinase C dependent pathways, therefore, were necessary for secretion.

We are currently screening the library of defective RBL cell clones (isolated by Dr. Reuben Siraganian). So far we have identified two clones of potential interest. One clone exhibits phosphatidylinositide breakdown and secretion but no calcium signal in response to antigen. This clone may be

highly dependent on the phosphatidylinositide/kinase C pathway. One clone, previously unresponsive to antigen, is now partially responsive. Comparisons between the responsive and nonresponsive state of this clone may, therefore, be instructive. Further work will be directed towards identifying defects in enzyme components of the signal cascade, and intracellular protein phosphorylation patterns. Thus far we have found substantial phospholipase C activity in all clones, both defective and responsive ones. Evaluation of the new Ca^{2+} probe, fura 2, which is 50 times more sensitive than quin 2, is now underway.

Purification of Isozymes of Cytochrome P-450

Most chemically unstable metabolites are formed in the body by a group of enzymes collectively known as cytochrome P-450. Although various laboratories have isolated many of the isozymes of cytochrome and in some instances established their amino acid sequence, the extent to which the activities of the purified isozymes in reconstituted systems reflect the activities of the isozymes in microsomes and in vivo is debatable.

In the past, we have reported that a monoclonal antibody developed in H. Gelboin's Laboratory (NCI) almost completely inhibited the metabolism of R-propranolol by liver microsomes from rats treated with @-naphthoflavone. However, the relative rates of formation of the metabolites of R-propranolol by the liver microsomes were markedly changed when the microsomes are solubilized by cholate and supplemented with NADPH cytochrome P-450 reductase. Moreover, the relative rates of formation of the metabolites by the antibody-sensitive isozymes in reconstituted systems were markedly different from those in microsomes; indeed no combination of the isozymes could have possibly resulted in the relative rates obtained with liver microsomes. During the past year we have discovered that the reconstituted systems produce superoxide and hydrogen peroxide which accelerate the N-dealkylation of R-propranolol and rapidly destroy another metabolite, 4-hydroxypropranolol. When the reconstituted systems are supplemented with superoxide dismutase and catalase, the relative rates of formation of the metabolites approached those obtained with liver microsomes.

Reconstituted cytochrome P-450 systems. To continue our exploration of reconstituted systems, we have purified several isozymes of cytochrome P-450 from rat liver microsomes. So far, we have isolated 6 isozymes from liver microsomes of rats treated with 3-methylcholanthrene, 8 isozymes from liver microsomes of rats treated with phenobarbital and 5 isozymes from liver microsomes of untreated male rats. These preparations represent at least 11 different isozymes, 3 or 4 of which appear to be isozymes not previously isolated in other laboratories. We are attempting to develop polyclonal antibodies against all of these forms, and have succeeded in developing antibodies against many of them. In addition, we have isolated 4 proteins from rat liver mitochondria, which may be isozymes of cytochrome P-450. The absorbancies of the carbon monoxide complexes at 450 nm are very low, however, and thus the preparations from mitochondria contain only trivial amounts of enzymatically active cytochromes P-450.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00617-09 LCP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The mechanism of carrageenan induced inflammation in rat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Theresa N. Lo	Research Chemist	LCP	NHLBI
Other:	Wilford F. Saul	Chemist	LCP	NHLBI

COOPERATING UNITS *(if any)*

Serrine S. Lau, Senior Staff Fellow, Lab. Exp.Therap. and Metabolism,DTP, DCT. NCI

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cells isolated from the pleural cavity of rat consisted mainly of macrophages, mast cells, eosinophils, lymphocytes and mesothelial cells. Isolated pleural cells labeled with [14C]-arachidonic acid released substantial amounts of radiolabel upon exposure to pharmacologically relevant concentration of carrangeenan (1-100 ug/ml). The time course of release was also compatible with the in vivo inflammatory action of carrageenan. An inhibitor of phospholipase A2 (i.e., p-bromophenacyl bromide) but not of arachidonate cyclooxygenase (i.e., indomethacin) inhibited the release in a dose-dependent manner. Analysis (by HPLC and radioimmunoassay) of the released products indicated that more than 90% of the radiolabel was arachidonic acid. Carrageenan also caused significant release of lactate dehydrogenase (LDH) from pleural cells. Release of LDH generally paralleled release of radiolabel although release of radiolabel preceded that of LDH. That the two events did not bear a cause-effect relationship, however, was demonstrated by the failure of p-bromophenacyl bromide to block release of LDH.

GPO 914-918

Project Description:

Objectives: In past years, we have studied the mechanism underlying the acute inflammatory reaction in response to the injection of carrageenan into the pleural cavity of the rat. We found that carrageenan induced a progressive accumulation of a protein-rich exudate which contained large numbers of neutrophils and that indomethacin inhibited exudation. These and other reports suggest that arachidonate metabolites mediate the exudation of plasma protein as an early event in the inflammatory response. A plausible inference is that carrageenan exerts its effect by interacting with specific cell population(s) within the pleural cavity, thereby causing the release of vasoactive mediators, such as the metabolites of arachidonic acid. This year we have isolated cells from the pleural cavity of the rat and examined the interaction of the isolated cells with carrageenan. We found that isolated pleural cells labeled with [^{14}C] arachidonic acid ([^{14}C] ARA) released substantial amounts of arachidonic acid upon exposure to carrageenan. Although the release of arachidonic acid was accompanied by parallel release of cytosolic lactate dehydrogenase (LDH), the two events appeared not to be casually related.

Methods Employed: The procedures used in the isolation and labeling of pleural cells and the measurement of the released radiolabel were described in detal in last year's report (ZO1 HL 0617-8 LCP). Labeling of other cell cultures was done in an identical manner.

Lipid analysis. Cellular lipids were extracted as follows: A mixture of chloroform and methanol was added to the cells to form a monophasic mixture ($CHCl_3/CH_3OH/H_2O$:1/2/0.8). The mixture was separated into two phases by addition of chloroform and 2M KCl in H_2O. Unlabeled standards were added to the chloroform layer, which was then dried under nitrogen and chromatographed on silica gel plates (solvent system: $CHCl_3/CH_3OH/CH_3COOH/H_2O$ = 75/45/3/1, v/v). Standards were visualized by iodine vapor before they were scraped from the plate. The components were then assayed for radioactivity.

Assay of lactate dehydrogenase activity and plasma membrane integrity. The activity of LDH was analyzed by a Sigma No. 226 UV assay kit. The integrity of cell membranes was assayed by a staining procedure using ethidium bromide (EB) and fluorescein diacetate (FDA). Viability of cells was indicated by a green cytoplasmic fluorescence; cells with damaged membrane were indicated by orange nuclear fluorescence with EB. At least 500 cells per sample were counted.

Major Findings: Incorporation of [^{14}C] ARA into cellular lipid and distribution of radioactivity in pleural cells. Incorporation of radiolabel was related to concentration of [^{14}C] ARA and near maximal incorporation (40,001 $\pm$ 4,846 dpm/10^6 cells) was achieved with 1.5 uM of [^{14}C]ARA. Of the incorporated label, 65 $\pm$ 1% was incorporated into phosphatidylcholine, 14 $\pm$ 1% into phosphatidylethanolamine, with the remaining distributed between phosphatidylinositol, phosphatidylserine and the neutral lipids.

Relationship between carrageenan-stimulated release of radiolabel and LDH. Within 5 min of addition of carrageenan, significant release of radiolabel from the pleural cells was observed (1011 ± 39 dpm/0.36 x 10^6 cells for the control; 1371 ± 94 dpm/0.36 x 10^6 cells for carrageenan-treated cells). Release continued for at least 90 min; but the maximal rate of release occurred between 30 and 45 min. Release was proportional to the number of cells over the range of 0.25 10^6 cells. There was little release at 15 min, but by 30 min release of LDH was significant (maximum release: 23 ± 2% of total cellular LDH activity). In the absence of carrageenan, no significant release of LDH was observed. Stimulated release of ^{14}C-label and LDH occurred with as little as 1 ug/ml of carrageenan and was maximum at 25-100 ug/ml. Although release of LDH paralleled release of ^{14}C-label in response to different concentrations of carrageenan, at the lower concentrations, the two responses could be dissociated in that a steeper concentration-response curve was obtained with the ^{14}C release. Staining with fluorescein diacetate and ethidium bromide indicated that only 75 ± 4% (n=2) of the pleural cells remained viable upon exposure to 100 ug/ml of carrageenan for 45 min (viability of untreated cells: 92 ± 2%).

No cause effect relationship exists between carrageenan-stimulated release of radiolabel and LDH: Membrane damage did not appear to be a consequence or cause of the release of radiolabel. Addition of 10 uM p-bromophenacyl bromide blocked stimulated release of radiolabel by 46% but did not prevent LDH release. In contrast, indomethacin (40 uM) caused a modest enhancement of radiolabeled release without evoking any effect on LDH release.

Comparison with adherent pleural cells. The pattern of [^{14}C]-ARA incorporation into the various classes of cellular lipid in pleural cell suspensions and adherent cells was similar. The adherent cells also responded to the same range of carrageenan concentration, i.e. 1-100 ug/ml. Differential cell counts indicated that 65-70% of the adherent cells are macrophages.

Specific of response. Among the four cell types tested (namely primary cultured rat submandibular gland cells, rat basophilic leukemia cells, human mesothelioma cells, and rat pleural cells) only the pleural cells responded to carrageenan. All four cell populations, however, did respond to 0.5-2 uM of the calcium ionophore A23187. Neither compound 48/80 nor dextran T10 stimulated the release of radiolabel from pleural cells labeled with [^{14}C]-ARA.

Significance to Biomedical Research and the Program of the Institute. Although carrageenan has been widely used as an experimental inflammatory agent, the detailed mechanism by which the agent induces acute inflammatory responses in the animal is not known. It is generally assumed that activation of arachidonic acid metabolism plays a role in the inflammatory process, because the responses can be suppressed by indomethacin and other nonsteroidal anti-inflammatory drugs. Our findings that carrageenan can stimulate release of arachidonic acid from pleural macrophages, a cell population normally present in the body cavity, imply that activation of phospholipase A_2 in these cells is an early event in the carrageenan-induced inflammatory response. Likewise, carrageenan has been reported to be

toxic to macrophages though at much higher concentrations (500-2000 ug/ml) than those used here. Our data showed that the cytotoxic effect of carrageenan may be unrelated to release of labeled arachidonic acid. Thus, the primary effect of carrageenan as an inflammatory agent is probably on the activation of phospholipase A_2, an enzyme that has been implicated in other experimental models of inflammation. However, its cytotoxic action may initiate further inflammatory reactions.

Proposed Course of Project: Our studies suggest that activation of phospholipase A_2 (the enzyme involved in the cleavage of arachidonic acid from phospholipids) in pleural cells is an early event in the inflammatory response to carrageenan in vivo. Other work in the laboratory indicates that activation of phospholipase C is an obligatory step leading to degranulation of mast cells. Both enzymes are activated by elevated cytosolic calcium concentration. It has been reported that the main substrate for phospholipase A_2, i.e. phosphatidylcholine, resides preferentially on the outer surface of the plasma membrane, whereas the putative substrate for phospholipase C, i.e. phosphatidylinositol, resides in the cytoplasmic portion of the membrane. Further work will investigate the differences in mechanism by which these two enzymes are activated.

Publication:

Lo, T.N., WoldeMussie, E., Wilson, N., Wu, D. and Beaven, M.A.: Inflammatory response induced by intrapleural injection of antiserum to IgE in rat. An evalaution of the role of histamine. Biochem. Pharm., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00620-08 LCP

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of histamine synthesis and release in tissues

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Elizabeth WoldeMussie	Staff Fellow	LCP	NHLBI
Other:	Michael A. Beaven	Deputy Chief	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mast cells in rat peritoneal cavities have heterogeneous characteristics. They show variation in maturity as well as in their biochemical activities. Mast cells in all 4 stages of development were present in the cavity. Young cells (stages 1 and 2) contained little histamine but had high histamine synthetic activity. They were unable to retain most of their newly synthesized histamine. Mature cells (stages 3 and 4), on the other hand, contained more histamine and had lower histamine synthetic activity than did immature cells. The young cells were resistant to degranulating agents while the mature cells were fully responsive. In cultured rat basophilic leukemia (2H3) cells were similar to immature peritoneal mast cells in having high histamine synthetic activity, low histamine content and high rates of histamine turnover. Changes related to cell maturation were also evident in 2H3 cells. Histamine synthetic activity, histamine content and responsiveness to stimulants increased as cells approach 'S' phase of the cell cycle and then decreased as cells approached mitosis.

Project Description:

Objectives: It is well known that mast cells from different tissues within the same animal may have diverse properties. As described in this and previous reports we have shown that maturational changes result in heterogeneous populations of mast cells in a single tissue or within a clone (2H3 basophils) of cultured cells. Our ability to isolate and study cells at different stages of maturity has provided useful information on the mechanisms of histamine synthesis and storage in the intact mast cells. In this report we show that histamine content is related to and histamine turnover is inversely related to the number of intracellular granules and maturity of cells.

Methods Employed: Peritoneal cells were collected from male Sprague Dawley rats by injecting Hanks' medium into the peritoneal cavity and then separated into fractions of increasing cell sizes by elutriation (J.P.E.T. 224:620-626, 1983). Rat basophilic leukemia cells were cultured and maintained in supplemented minimum essential medium with Earle's balanced salt solution. They were removed from flasks by trypsinization and separated by elutration. The procedures have been described in detail in previous reports.

Histamine content of samples were determined by radioenzymatic assay. Histamine synthetic activity was assayed by measuring $^{14}CO_2$ released as a result of decarboxylation of ^{14}C (carboxyl) histidine (JPET 224:620-626, 1983).

Major Findings: Mast cells were categorized according to size and maturity (i.e. stage 1 to 4). Elutriated peritoneal mast cells were collected in fractions 3 to 12. Fractions 3 to 5 contained small immature Stage 1 mast cells, fractions 6 to 9 contained intermediate stage 2 and 3 cells and the large mature stage 4 cells were collected in fractions 10 to 12. Histamine content of these fractions ranged from 0.6 pg/cell for stage 1 cells to 12 pg/cell for stage 4 cells. Histamine synthetic activity, in contrast, was highest in fractions that contained predominantly stage 2 cells and, upon progression to fractions of larger cells, activity was found to decrease by 70-80%.

Studies with cell suspensions indicated that, in vitro, immature mast cells lacked the ability to store newly synthesized histamine which was continuously released into the incubation medium. However, as the cells matured they retained most of their newly synthesized histamine. The turnover rate of histamine was calculated to be 0.12/hr in cells in fraction 5 down to 0.05/hr or less in cells from fractions 8 to 11. Exposure to α-fluoromethylhistidine (10^{-5}M), which inhibited histamine synthetic activity in both immature and mature mast cells by > 90%, resulted in a marked decrease in rates of spontaneous release of histamine and histamine content of immature mast cells. These data provided further confirmation of rapid histamine turnover in immature cells. Such effects were not observed in mature cells.

The responsiveness of the mast cells to stimulants such as compound 48/80 and the Ca^{2+} ionophore A23187 increased with the maturity of the cells. Younger cells were resistant and mature cells were fully responsive to these stimulants. The above findings indicated that the systems for storage and secretion of histamine are expressed or become functional at a relatively late

stage of mast cell maturation. Recent morphological studies suggest that the capacity for histamine storage is related to the number of intracellular granules and the extent of sulfation of mucopolysaccharides within the granules.

The rat basophilic leukemia (2H3) cells were similar to the immature mast cells in many respects. Although they had high histamine synthetic activity, they had low histamine content (0.3-0.6 ug/cell) and they released histamine continuously into the medium. Calculated histamine turnover rates in 2H3 cell cultures were of the order 12 hr compared to 10 hr in immature peritoneal mast cells. Histidine labeling experiments suggested that, at isotopic equilibrium, 33% of the histamine was present in the cytosol and 66% was present in storage granules. Of possible relevance, the maximal response obtained with these cells with antigen or cross-linked IgE oligomers has been 60-65% net histamine release.

An apparent heterogeneity of the 2H3 cells was related to their cell cycle. Their activity (histidine uptake and histamine synthesis) as well as histamine content were highest just before the cells reached S phase and were greatly reduced as the cells approached cell division. Previous studies have shown also that the cells lose their ability to secrete histamine, but not their ability to generate intracellular signals, as they pass through mitosis (J. Cell Biol. 98: 2250, 1984). Histamine synthetic activity and responsiveness to secretory stimuli was restored as cells passed hrough G_1 phase of growth.

Significance to Biomedical Research and the Program of the Institute: In this study young mast cells were shown to be resistant to degranulating agents. As the sequence of biochemical events that lead to degranulation is unclear, studies of the development of the biochemical components of the secretory process during cell maturation may be instructive. The rapid turnover of histamine in immature mast cells and the transformed 2H3 cells has clinical implications. Lesions of immature or transformed mast cells resulted in frequent spontaneous inflammatory reactions and itching.

Proposed Course of Project: Further studies will focus more specifically on the biochemical changes related to cell maturation, for example, the ability to generate stimulatory signals in response to secretory stimuli.

Publications:

Beaven, M.A. and WoldeMussie, E.: Histamine in body fluids: Its measurements in different clinical states. New England and Regional Allergy Proceedings 5: 300-310, 1985.

WoldeMussie, E. and Beaven, M.A.: Heterogeneity of rat peritoneal mast cells and 2H3 leukemic basophils in relation to cell maturation and cell cycle. Invited presentation, Symposium on Mast Cell Heterogeneity, Ontario, Canada, Feb. 4-6, 1985, in press.

WoldeMussie, E. and Beaven, MA.: α-Fluoromethylhistidine: Kinetics of uptake and inhibition of histamine synthesis in basophil (2H3) cell cultures. Molecular Pharmacology, in press.

WoldeMussie, E., Aiken, D. and Beaven, M.A.: Changes in histidine uptake, histamine synthesis and histamine levels during the growth cycle of rat basophilic leukemia (2H3) cells. J. Pharm. Exptl. Therap. 232: 20-26, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00805-03 LCP

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Anti-P-450 monoclonal antibodies: Effect on drug metabolism in different tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Henry A. Sasame	Chemist	LCP	NHLBI
Other Investigator: James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NIH, NHLBI-IR-LCP, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major pitfall encountered with the study of R-propranolol metabolism of a purified cytochrome P-450 system was the destruction of 4-hydroxypropranolol formed during the incubation. The plausible cause of this distinction is primarily due to the production of superoxide ions and hydrogen peroxide in the incubation media of reconstituted system. The combined presence of catalase and superoxide dismutase blocked not only the destruction of 4-hydroxypropranolol (4-OHP) but also the increased rate of desisopropylpropranolol (DIP) Consequently, the DIP/5OH and 4OHP/5-OH ratios obtained with reconstituted systems containing purified cytochrome P-450 supplemented with superoxide dismutase and catalase approached the ratios obtained with intact microsomes. This observation indicates that extrapolating data obtained by the reconstituted systems to intact microsomes should be made with caution.

Project Description:

Objectives: In recent years, many isozymes of cytochrome P-450 have been isolated from liver by different investigators. Comparisons of the relative rates of product formation from different substrates by a given isozyme differ markedly from one laboratory to others. These interlaboratory discrepancies may be due in part to different conditions obtained with reconstituted systems. In our laboratory we have discovered that the ratios of three major metabolites of R-propranolol by a purified cytochrome P-450 from 3MC treated rats differed markedly from that by intact microsomes. We have undertaken a series of experiments which were designed to resolve this discrepancy.

Methods Employed: Conventional biochemical techniques have been employed. The separation of R-propranolol metaoblites was carried out in Waters Associated HPLC equipped with Schoeffel Fluorescent Detector.

Major Findings: 1) In the reconstitute systems, containing cytochrome P-450$_c$ and NADPH cytochrome P-450 reductase, 4-hydroxypropranolol was preferentially destroyed as it was generated from R-propranolol. The destruction of 4-hydroxypropranolol was caused by the combined effect of superoxide ions and hydrogen peroxide presumably produced during autoxidation of P-450 reductase. Scavengers for superoxide such ascorbic acid, glutathione, superoxide dismutase blocked this destruction in part but not completely. Likewise catalase itself blocked partially. The combination of superoxide dismutase and catalase, however, completely blocked the destruction when the reconstituted systems were supplemented with catalyse and superoxide dismutase the relative rates of formation of 4-hydroxypropranolol, 5-hydroxy propranolol and DIP approached those obtained with microsomes.

2) Other probable oxidants such as hydroxy radicals, or Weiss reaction (EDTA + Fe^{++}) were ruled out by the fact that the antihydroxy radical agent, diethylene triamino pentacetic acid failed to block this destruction. In addition, without the presence of EDTA in the media there was far less formation of all three major metabolites.

3) The amount of destruction of 4-hydroxypropranolol depended on the amount of NADPH cytochrome P-450 reductase added the reconstituted system and the time of incubation.

4) The excess superoxide ions and hydrogen peroxide formed in the reconstituted system as well as in the cholate solubilized microsomes supplemented with NADPH cytochrome P-450 reductase increased the rate of formation of deisoproply propranolol. The increase was blocked by addition of catalase. Xanthine oxidase and hypoxanthene dealkylates R-propranolol only in the presence of cytochrome P-450 which acts as a peroxidase. The dealkyaltion was effectively blocked by combination of superoxide dismutase and catalase.

Significance to Biomedical Research and to Program of the Institute: A major objective of purifying isozymes of cytochrome P-450 has been to determine the substrate specificity and the pattern of product formation of the isozymes in

reconstituted systems. The project illustrates one of the pitfalls that occur with this approach.

Proposed Course of Project: The possibility that superoxide and hydrogen peroxide generated in reconstituted system may oxidize other substrates and metabolites will be persued.

Publciations: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00937-03 LCP

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biochemical mechanisms of mast cell degranulation: PI breakdown and Ca signal

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Kazutaka Maeyama	Visiting Fellow	LCP	NHLBI
Others:			
Michael A. Beaven	Deputy Chief	LCP	NHLBI
Lance R. Pohl	Pharmacologist	LCP	NHLBI

COOPERATING UNITS *(if any)*

Dr. Henry Metzger and Dr. Robert Hohman, NIADDK, Arthritis and Rheumatism Branch

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Breakdown of phosphatidyl inositol (PI) and related phosphoinositides appear to be an early event in IgE mediated degranulation of 2H3 cells since it occurs even when degranulation is prevented by blocking the influx of calcium (J.Biol.Chem. 259:7129;7137,1984). The relationship between IgE receptor cross-linking and PI breakdown was examined in the present studies by use of covalently cross-linked IgE polymers. As reported previously (J.Immunol.125:701, 1980), higher oligomers were more active in stimulating histamine release than trimers and trimers more so than dimers. Monomers were inactive. Both PI breakdown and increase in cytosolic calcium levels which were obtained with occupancy of several hundred receptors, were related to the number of receptors occupied. These responses were correlated with release of histamine. However, when histamine release reached a maximum, further breakdown of PI and increases in calcium were observed when additional receptors were aggregated. In the presence of 30% heavy water the rates of both breakdown of PI and release of histamine were increased up to 2.5-fold. Our studies indicate that 1) breakdown of PI is stoichiometrically correlated with the aggregation of receptors, 2) the maximal ability of the receptors to induce release of histamine is not limited by their capacity to induce breakdown of PI and 3) heavy water-well known to enhance secretion-may act at an early stage in the transduction of membrane signal(s) to enhance histamine release.

Project Description:

Objectives: Release of histamine and other inflammatory mediators from tissue mast cells and blood basophils is the primary event in a variety of acute allergic reactions. The reactions encompass a broad spectrum of clinical disorders which range from the mild discomfort of hay fever to more severe problems of asthma and in some instances anaphylactic shock and death. The use of chemical antagonists against all active substances secreted by mast cells and basophils is not a practical approach because of the diversity of active substances involved. Design of more effective therapeutic approaches would be facilitated when the process of mast cell secretion is properly understood.

Transient increases in cytolic Ca^{2+} levels through influx of Ca^{2+} ions across the cell membrane or Ca^{2+} mobilization from internal stores was thought to be the critical event for mast cell degranulation, but such increases had never been demonstrated directly. Studies were started two years ago by M.A. Beaven in Dr. J.C. Metcalfe's laboratory (University of Cambridge, England) to quantitate changes in intracellular cytosolic Ca^{2+} concentrations ($[Ca_i^{2+}]$) in stimulated cells by use of the fluorescent Ca^{2+} probe, quin 2. For convenience, studies were conducted with a histamine releasing clone (2H3) of the rat basophil leukemic (RBL) cell line. These studies showed that histamine release was directly dependent on increases in $[Ca_i^{2+}]$ (J.Biol.Chem. 259:7129,1984) and was associated with extensive (up to 70%) breakdown of membrane phosphoinositides (PI breakdown) to form water soluble inositol phosphates and diacylglycerol (DAG) which accumulated in the cell cytosol (J.Biol.Chem. 259:7137, 1984). The calcium signal and PI breakdown was totally dependent on the supply of external Ca^{2+} (Ca_i^{2+}), intracellular ATP and antigen. Both responses rapidly ceased once cell cultures were deprived of any one of these 3 factors. Both responses could be uncoupled from histamine release by arresting cells in mitosis (J. Cell Biol. 98:2250,1984) or by addition of Zn^{2+}. Subsequent studies in this laboratory provided additional evidence to support the view that PI breakdown was intimately associated with Ca^{2+} mobilization in 2H3 cells (see last year's report) as appears to be the case in other secretory cell systems (Berridge, Biochem. J. 220:345, 1984).

It is now apparent, however, that PI breakdown may initiate at least two stimulatory signals in 2H3 cells. One (this report) is the influx of Ca^{2+} ions across the plasma membrane; the other is activation of protein kinase C by DAG (report No. Z01 HL 00973-01 LCP). As reported here, the relationships between PI breakdown and Ca^{2+} influx has been studied by use of cross-linked dimers, trimers and higher oligomers of IgE F_c fragments. These preparations stimulate histamine release by cross-linking directly IgE receptors on the plasma membrane (J.Immunol.125:701,1980) in contrast to antigen which promotes aggregation of 2 or perhaps more IgE receptors by cross-linking IgE antibody attached to the IgE receptor. The use of oligomers allows us to quantitate the extent of receptor aggregation and to examine the possibility that agents known to suppress histamine release may do so by modifying the relationship between receptor aggregation and generation of intracellular signal(s).

Methods Employed: Cell culture procedures and techniques for measurement of $[Ca_i^{2+}]$ by quin 2, PI breakdown and histamine release have been described in detail in previous publications (J.Biol. Chem. 259:7129 and 7137, 1984). The cellular pools of phosphoinositides were labeled by overnight incubation of cells with ^3H-labeled inositol. The parent membrane phospholipids; phosphotidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-biphosphate (PIP_2) were separated from their water soluble cleavage products, inositol 1-monophosphate (IP), inositol 1,4-bisphosphate (IP_2) and inositol 1,4,5-triphosphate (IP_3) by solvent extraction. The individual phosphoinositides and inositol phosphates were separated by chromatography on Dowex-formate columns or thin layer plates as previously described. The identity of a previously unidentified product, inositol 1,2-cyclic monophosphate was confirmed by comparison with the chemically synthesized product (see results).

Rat myeloma (IR 62) IgE was covalently cross-linked with dimethyl suberimidate and then separated into the monomeric, dimeric, trimeric and higher oligomeric forms by gel electrophoresis (J.Immunol. 125:701,1980). The extent of IgE receptor binding was determined by addition of excess ^{125}I-radiolabeled IgE and the numbers of unoccupied receptors thereby determined.

Major Findings: 1) Relationship between PI breakdown, Ca signal and histamine release with various concentrations of oligomeric IgE preparations. As was predicted, the monomeric IgE preparation evoked no response in 2H3 cells at any concentration tested. The higher oligomers (i.e. 4 or more F_c fragments) were more active in stimulating PI breakdown, increases in $[Ca_i^{2+}]$ and histamine release than the trimers and trimers more so than the dimers. With the trimer and higher oligomers measurable responses were obtained with occupancy of as little as 100 or so receptors (out of 450,000) per cell. Both PI breakdown and increases in $[Ca_i^{2+}]$ were highly correlated (irrespective of whether dimer, trimer or higher oligomer were used) and were related to the number of receptors occupied. With occupancy of less than 10,000 receptors per cell histamine release was correlated with the extent of PI breakdown and Ca^{2+} signal. However, histamine release reached a maximum with occupancy of twenty to thirty thousand receptors although PI breakdown and Ca^{2+} signal continued to increase in relation to numbers of receptors occupied until all receptors were saturated.

2) Correlation between PI breakdown and Ca^{2} + signal: Time course studies. Previous studies have shown that in response to a given concentration of stimulant rates of histamine release were linearly related to the extent of increase in $[Ca_i^{2+}]$. This relationship held true when $[Ca_i^{2+}]$ was increasing or was decreasing once a maximal response had been obtained. Similar studies, in which $[Ca_i^{2+}]$ and PI breakdown was measured simultaneously in the same batch of cells, indicated a more complex relationship. A linear relationship was obtained when rates of PI breakdown on an arithmatic scale was plotted against $[Ca_i^{2+}]$ on a logarithmic scale. This relationship suggested that PI breakdown stimulated increases in $[Ca_i^{2+}]$ through at least two, possibly synergistic, pathways.

3) Enhancement of responses by heavy water (D_2O). The enhancement of histamine release in the presence of D_2O is a well documented phenomenon. This phenomenon has been attributed to stabilization of microtubules.

In our hands, D_2O was shown to enhance PI breakdown, the Ca^{2+} signal and histamine release to the same extent. This enhancement, which was evident with a wide range of the concentrations of trimer and higher oligomer, was related to concentration of D_2O over a range of 0-45% D_2O. Whatever the concentration of D_2O or stimulant, the correlation between rates of PI breakdown and increase in Ca_i^{2+} was unchanged. The data suggested that the increased Ca^{2+} signal (and histamine release) was a consequence of increased PI breakdown. An additional PI product, tentatively identified as inositol 1,2-cyclic monophosphate (see below), was produced upon stimulation of cells in the presence of D_2O.

4) Identification of inositol 1,2-cyclic monophosphate (IcP). A minor radioactive product isolated from Dowex-formate columns had, based on previously published data, been identified as glycerophosphoinositol (GPI). The peak was increased when cells were stimulated in the presence of D_2O. When extractions were performed under acid instead of neutral conditions the yield of this radioactive product was increased even further. Based on chromatographic data, the radioactive product was tentatively identified as IcP. Incubation of 2H3 cells with the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), resulted in increased yields of IcP and decreased yields of IP. This effect was dependent on concentration of IBMX over the range of 1 to 100 µg/ ml. With 100 µg/ml IBMX IcP accounted for 42% of the total inositol phosphates generated upon cell stimulation.

Myo-inositol 1,2-cyclic phosphate was synthesized by condensing myo-inositol 2-phosphate with dicyclohexylcarbodiimide. The structure of the product was confirmed by negative ion fast ion bombardment mass spectrometry. Properties of the synthetic and endogenous products are now being compared. The data implied that hydrolysis of PI proceded thus, PI → IcP → IP.

Significance to Biomedical Research and the Program of the Institute. We have established that 1) breakdown of PI is stoichiometrically related to the numbers of IgE receptors aggregated, 2) rates of PI breakdown are related to exponential increases in $[Ca_i^{2+}]$, a possible indication of multiple synergistic pathways in the signal cascade mechanism, 3) D_2O and other treatments known to modulate histamine release (e.g. temperature changes, ATP depletion - see other reports) may do so by influencing mechanisms related to PI breakdown and 4) the maximal capacity of 2H3 cells to release histamine is not limited by the numbers of available IgE receptors or capacity of cells to induce PI breakdown. As 2H3 cells are totally dependent on Ca^{2+} influx for stimulation (see previous reports), our progress thus far provides a useful basis, to examine critically the question of whether or not PI breakdown promotes Ca^{2+} influx. This is in contrast to other cell systems in which Ca-dependent responses can be mediated by release of Ca^{2+} ions from intracellular stores by IP_3 (see project report Z01, HL 00973-01 LCP).

Proposed Course of Project: Future work will include: 1) rigorous identification of the putative IcP; 2) study of the effect of IcP (and other inositol phosphates) on Ca^{2+} mobilization and PI breakdown in permeabilized cell systems; 3) determination of whether PI breakdown and Ca signal are transient or sustained events once receptors are aggregated (by removal of excess oligomer) i.e. is additional receptor occupancy required to sustain events; 4) characterization and study of distribution of phospholipase C (the enzyme responsible for PI hydrolysis) in 2H3 cells.

Publications:

1. Soll, A.H., Amirian, D.A., Thomas, L.P., Park, J., Beaven, M.A. and Yamada, T.: Gastrin receptors on nonparietal cells isolated from canine fundic mucosa. Am. J. Physiol., 247:G715-723, 1984.

2. Beaven, M.A., WoldeMussie, E., Moore, J.P., Rogers, J. and Smith, G.A.: Studies of the calcium signal (quin-2 fluorescence), phospholipid turnover and histamine release in basophil leukemic (2H3) cells. In Frontiers of Histamine Research (Ed. C.R. Ganellin and J.C. Schwartz), Advances in the Biosciences, Vol. Pergamon Press, in press.

3. Nabika, T., Velletri, P.A., Lovenberg, W. and Beaven, M.A.: Increase in cytosolic calcium and phosphoinositide metabolism induced by antigotensin II and [arg]vasopressin in vascular smooth muscle cells. J. Biol. Chem. 260: 4661-4670, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00958-03 LCP

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Drug-induced cardiotoxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Vilas Shirhatti	Vist. Fellow	LCP	NHLBI
Others:				
	James Reese	Guest Worker	LCP	NHLBI
	G. Krishna	Chief, Section	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cultured rat cardiac myocytes have been used as a model to study the possible role of oxidative stress in the cardiotoxicity induced by anthracycline anti-cancer drugs. Changes in the levels of oxidized glutathione levels (GSSG) inside the cell, leakage of reduced glutathione (GSH) and glutathione oxidized (GSSG) from the cells were mainly used as a parameter of oxidative stress. Adriamycin and daunomycin caused a dose and time dependent increase in leakage of glutathione. About 70 to 80% of the glutathione that leaked out of cells was found to be in the reduced form. A selective inhibitor of GSH reductase, bis-chloro ethyl nitroso urea (BCNU) markedly potentiated the daunomycin induced toxicity. 4'-Deoxyadriamycin, which has been shown not to form free radicals that are prerequisites for oxidative stress, also caused a time and dose dependent leakage of GSH without much change in GSSG in the cells. Calcium Ionophore A2318, which does not form any free radicals, also caused a time dependent increase in leakage of GSH from cells without affecting the intracellular GSSG levels.

347

GPO 914-918

Project Description:

Objectives: The main objective of this study is to elucidate the mechanisms responsible for the cardiotoxicity of the anthracycline anti-tumor drugs of which adriamycin is the archetype. The major side effect of these drugs is their ability to induce a dose dependent cardiomyopathy which results in cardiac failure. In the previous years we have reported the developement of a cardiac myocyte culture which could be employed as an effective model for studying such toxicity mechanisms. Leakage of LDH, CPK and reduction in cellular ATP, have been employed previously as parameters for cardiotoxicity. In this study we have investigated the importance of oxidative stress in the toxicity induced by these drugs which are quinones and thus are capable of generating free radicals leading to generation of superoxide and other active oxygen metabolites.

Methods Employed: The methods of cell isolation culture and the modifications of the methods have been described in the last project report. Leakage of LDH and CPK, reduction in cell ATP, and leakage of [^{14}C] 5'-AMP were measured as described earlier. GSH and GSSG in cells as well as the medium were measured by enzymatically coupled cycling systems using GSSG reductase, NADPH and 5,5'-dinitro, 3,3'dithiobenzoic acid (Ellman reagent).

Major Findings: Cultured rat cardiac myocytes were used as a model for studying the possible role of oxidative stress in the cardiotoxicity induced by anthracycline. Changes in the levels of oxidized glutathione levels (GSSG) inside the cell, leakage of reduced glutathione (GSH) and oxidized glutathione (GSSG) from the cells were mainly used as a parameter of oxidative stress. Adriamycin and daunomycin caused a dose and time dependent increase in leakage of glutathione. About 70 to 80% of the glutathione that leaked out of cells was found to be in the reduced form. A selective inhibitor of GSH reductase, bis-chloro ethyl nitroso urea(BCNU) markedly potentiated the daunomycin-induced toxicity. 4'-Deoxyadriamycin which has been shown not to form free radicals that are prerequisites for oxidative stress also caused a time and dose dependent leakage of GSH without much change in GSSG in the cells. Calcium Ionophore A23187, which does not form any free radicals, also caused a time dependent increase in leakage of GSH from cells without affecting the intracellular GSSG levels.

Both adriamycin and daunomycin increased leakage of glutathione from cardiac myocytes in a time and concentration dependent fashion. Even though these drugs decreased GSH levels within the cell, GSSG levels were not increased to any great extent. Moreover, the glutathione that leaked into the medium contained GSH in greater proportion than GSSG (7:3). When the cardiac myocytes were preincubated with BCNU, which inhibits GSH reductase, there was a small increase in GSH leakage. When these cells were incubated with daunomycin, there was a marked potentiation in GSH release from the cells. The intracellular GSSG levels increased only slightly. BCNU also potentiated markedly enzyme and nucleotide release induced by daunomycin.

4'-Deoxyadriamycin which has been shown not to produce free radical intermediates, increased glutathione release to the same extent as daunomycin. Moreover, calcium ionophore A23187 which does not generate free radical intermediates as daunomycin, also markedly increased GSH release from cardiac myocytes. FCCP an uncoupler of oxidation phosphorylation, also increased glutathione release. All these agents did not markedly alter intracellular levels of GSSG. These findings indicate that oxidation of GSH may not be a prerequisite for the leakage of GSH from cardiac myocytes.

Significance to Biomedical Research and the Program of the Institute: The major finding in this study is that oxidative mechanism is not necessary for the loss and leakage of GSH by anthracycline anti-tumor drugs. This confirms one previous finding that superoxide generation *per se* is not a requisite for the cardiotoxicity induced by these drugs. The measurement of GSSG, however, may be useful parameter for the study of superoxide generation within the cell.

Proposed Course of Project: The mechanism by which both adriamycin and daunomycin induce leakage of GSH from cardiac myocytes will be investigated using a number of analogs of these drugs which have been shown to have varying degrees of cytotoxicity. Since mitochondrial GSH levels may play an important role in oxidative phosphorylation, the effect of anthracycline on mitochondrial GSH levels will be compared with the effect on ATP synthesis.

Publications:

1) Shirhatti, V., and Krishna, G.: A simple and sensitive method for monitoring drug-induced cell injury in cultured cells. Analytical Biochemistry, in press.

2) Shirhatti, V., and Krishna, G.: Beating cardiac myocytes maintained in culture: A model for screening the study of mechanism of drug-induced cardiotoxicity. In: In Vitro Toxicology, Vol. 3, Goldberg, A. (ed.), Liebert Publishers, Inc., New York, New York., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00962-03 LCP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunological studies on the mechanism of halothane-induced hepatotoxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Hiroko Satoh	Vist. Assoc.	LCP	NHLBI
Others:			
Lance R. Pohl	Pharmacologist	LCP	NHLBI
James R. Gillette	Chief	LCP	NHLBI
Helen W. Davies	Staff Fellow	LCP	NHLBI
John W. George	Chemist	LCP	NHLBI
Kaori Maeda	Vist. Fellow	LCP	NHLBI

COOPERATING UNITS *(if any)*

Dr. Tamiko Takemura and Dr. V.J. Ferrans, Ultrastructure Section, Pathology Branch, NHLBI; Sandra Jelenich, Anesthesiology Section, Clinical Center, NIH; James Neuberger, King's College Hospital, London, England.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.3	1.9	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously reported that halothane's reactive oxidative metabolite, trifluoroacetyl halide forms trifluoroacetylated (TFA) covalent adducts within and on the outer surface of hepatocytes, when halothane is administered to rats. These adducts have now been further characterized by various immunochemical procedures and their relationship to halothane-induced hepatotoxicity is being investigated. The major TFA adduct within the cell has been identified as a 54 kD form of microsomal cytochrome P-450. A 54 kD TFA protein was also detected in the plasma membrane fraction of the cell. Immunoperoxidase staining of Western blots of plasma membrane proteins with specific antibodies revealed that cytochrome P-450-54 kD and cytochrome P-450 reductase were present in this fraction of the cell. Immunoelectron microscopic examination of intact hepatocytes confirmed that cytochrome P-450 was a component of the plasma membrane. TFA adducts were also detected in microsomes from patients that were administered halothane. Moreover, the sera of two of six patients that had the fulminant form of halothane-induced hepatitis, contained anti-TFA antibodies. These results show unequivocally that cytochrome P-450 and cytochrome P-450 reductase are plasma membrane components. This importantly implies that the toxicity of many drugs maybe due to the formation of reactive and toxic metabolites at this site, where they may cause death either by an acute injury or by an immunologically-based mechanism. In the case of halothane, its reactive trifluoroacetyl halide metabolite maybe produced, at least in part, in the plasma membrane where it forms TFA adducts. In susceptible individuals, these adducts maybe recognized by the immune system as foreign antigens, resulting ultimately on second exposure in an immunologically-based hepatotoxicity.

GPO 914-918

Project Description:

Objectives: The intent of this investigation is to determine the molecular basis of halothane-induced hepatotoxicity. The methodologies developed in this study should serve as a model for investigating other drug-induced toxicities that have an immunological basis.

Method Employed: The following immunological techniques with anti-TFA, anti-cytochrome P-450-52 kD and -54 kD, and anticytochrome P-450 reductase antibodies were used to characterize TFA adducts in the livers of halothane treated rats and humans and anti-TFA antibodies in human serum: Immunoperoxidase staining of constituent proteins of rat and human liver tissue; immunoaffinity chromatography; enzyme linked immunoabsorbent assay (ELISA); and immunoperoxidase electron microscopy.

Major Findings: Four hours after the adminstration of halothane to phenobarbital pretreated rats, subcellular fractions of liver were isolated and the proteins in the fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred electrophoretically to nitrocellulose sheets, (Western blots) and immunochemically stained with anti-TFA antibodies. The microsomal fraction contained the highest level of TFA adducts. Its major TFA component was immunochemically identified as a phenobarbital inducible form of cytochrome P-450 (54 kD), whereas the other observed TFA protein fraction (59 kD) was not identified. The plasma membrane fraction also contained a 54 kD trifluoroacetylated adduct, which was immunochemically related to the 54 kD cytochrome P-450. Microsomes from untreated rats that were administered halothane contained only the 59 kD trifluoroacetylated protein fraction. The specificity of the immunochemical staining for the bound oxidative metabolite of halothane was confirmed by the finding that rats treated with deuterated halothane had considerably less stained liver proteins than those treated with halothane.

Immunochemical staining with anti-TFA antibodies of Western blots of human microsomes isolated from biopsy samples approximately 4 hr after halothane administration revealed the presence of TFA adducts. The number of TFA adducts seen in the human liver samples varied from as low as 1 major adduct to as high as 3 major adducts. The apparent molecular weights of these TFA components were in the range of approximately 45 to 60 kD.

ELISA analysis of 6 serum samples from patients that were purported to have had an episode of halothane-induced immunohepatocellular damage, indicated that 2 of the patients contained anti-TFA antibodies in their serum.

Immunochemical staining with anti-cytochrome P-450 and anticytochrome P-450 reductase antibodies of Western blots of liver plasma membrane fractions isolated from rats treated with phenobarbital showed the presence of immunoreactive cytochrome P-450-54 kD and cytochrome P-450 reductase. Cytochrome P-450-54 kD was also detected in the plasma membrane of intact hepatocytes by immunoperoxidase electron microscopy.

Significance to Biomedical Research and Program of the Institute: Many drugs, such as halothane, cause rare but fatal toxicities that have often been classified as idiosyncratic or hypersensitivity reactions. Although the basis of these toxicities remains obscure, it has been hypothesized that they often may be due to tissue sensitization produced by a metabolite. We believe that our studies on the immunological basis of halothane-induced fulinant hepatotoxicity may serve as a model approach to investigate these types of toxicities. For example, we have found that halothane's reactive oxidative metabolite, CF_3COX, can form adducts with cellular proteins, including components of the plasma membrane, when halothane is administered to rats and humans. In some patients, these adducts appear to elicit a humoral response, which might be responsible, at least in part, for the observed hepatocellular damage.

Our findings that cytochrome P-450 and cytochrome P-450 reductase are plasma membrane constituents has far reaching implications. It indicates that reactive metabolites may be generated in the plasma membrane where they can cause direct acute tissue damage. Alternatively, the metabolites produced in the plasma membrane may alter the antigenic nature of the plasma membrane so that it becomes immunogenic, resulting ultimately in tissue sensitization and necrosis.

Proposed Course of Project: We intend to characterize further the halothane-induced immunogens that are responsible for eliciting the immune response and hepatocellular damage. Once these immunogens have been identified, they can be used in the development of ELISA methods for detecting patients that have been sensitized to halothane. The specific immunogens can also be used to develop animal models of the immunotoxicity so that the intimate cellular details of the immune response and ultimate cellular damage can be investigated. These studies can also be extended to other drugs that have been proposed to produce tissue damage by an immune mechanism. We also intend to characterize further the plasma membrane cytochrme P-450 system in order to determine its potential role in drug-induced toxicities.

Publications:

Satoh, H., Fukuda, Y., Anderson, D.K., Ferrans, V.J., Gillette, J.R. and Pohl, L.R.: Immunological studies on the mechanism of halothane-induced hepatotxicity: Immunohistochemical evidence of trifluoroacetylated hepatocytes. J. Pharm. Exp. Therap., in press.

Callis, A.H. Brooks, S.D., Waters, A.J., Gandolfi, Lucas, D.O., Pohl, L.R., Satoh, H., Sipes, I.G.: Evidence for a role of the immune response in the pathogenesis of halothane hepatitis. In: Molecualr mechanisms of anesthesia, Progress in Anesthesiology, Vol.3, Sheldon H. Roth (ed.), Raven Press, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00967-03 LCP

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of cytochrome P-450 turnover

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Helen W. Davies	Staff Fellow	LCP	NHLBI
Others:				
	Lance R. Pohl	Section Chief	LCP	NHLBI
	Hiroko Satoh	Vist. Assoc.	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI
	Kaori Maeda	Vist. Fellow	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Pharmacological Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	1.8	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously reported that during the catalytic cycle of rat liver cytochrome P-450 in vitro that the prosthetic heme group is destroyed and its degradation products become irreversibly bound to microsomal proteins. The mechanism of this degradation process also appeared to be intimately linked to cellular lipid peroxidation, since more degradation occurred when microsomes were incubated with carbon tetrachloride or with linoleic hydroperoxide, which initiate lipid peroxidation. We now report that the heme-derived degradation products found in vitro are preferentially bound to cytochrome P-450. Moreover, a similar degradation process also takes place after the administration of carbon tetrachloride to rats and it appears to result in the rapid loss of immunoreactive cytochrome P-450 from liver microsomes. These results suggest that the bound heme degradation products have 'tagged' the cytochrome P-450 adducts for catabolism by cellular proteases. If other xenobiotics cause similar effects, this may have general importance in the regulation of the activity of this important family of enzymes that not only metabolize foreign compouds, but also endogenous substrates such as steroids, prostaglandins, leucotrienes, and fatty acids.

HS 6040 (Rev. 1/84) GPO 914-918

Project Description:

Objectives: To determine the mechanisms of inactivation of cytochromes P-450 heme and apoprotein turnover.

Methods Employed: Two major phenobarbital (PB) inducible forms of cytochrome P-450 were purified from rat liver and antibodies were raised against these enzymes in female rabbits. Rat liver proteins and heme were prelabeled with ^{14}C sodium bicarbonate and ^{3}H delta amino-levulinic acid, respectively, before each experiment. The cytochromes P-450 were then destroyed either by incubating the isolated microsomes with CCl_4 or by administration of CCl_4 to rats one hour before the animals were killed. The immunochemical identification of cytochromes P-450 [^{3}H]heme-derived adducts were determined in the following manner. Microsomes were solubilized to a final concentration of 8 mg/ml in 50 mM potassium phosphate (pH 7.4) containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 M KCl, 1% (w/v) sodium cholate, and 0.2% (v/v) Emulgen 911 and an aliquot (25-30 "g) was mixed with anti-cytochrome P-450-52 kD or anti-cytochrome P-450-54 kD IgG to precipitate immunoreactive cytochrome P-450-52 or cytochrome P-450-54 kD proteins. After the immunoprecipitates were solubilized in 60 mM Tris-HCl (pH 6.8) containing 2.3% (w/v) SDS, 10% (v/v) glycerol, and 5% (v/v) mercaptoethanol, samples were analyzed for irreversibly bound [^{3}H]heme-derived label by acetone-HCl precipitation. The radiolabel precipitated by this procedure is associated only with covalently bound heme fragments. All experiments were repeated with normal IgG; the nonspecific binding was subtracted from the above results to give corrected levels of irreversibly bound [^{3}H]heme-derived label. Immunoreactive cytochrome P-450 was determined by single radial immunodiffusion.

Major Findings: Immunoprecipitation of microsomes with antibodies raised against two PB-induced forms of cytochrome P-450 established definitively that CCl_4-induced destruction of cytochrome P-450 results in heme-derived radiolabel bound irreversibly to cytochromes P-450. Eleven percent of the heme-derived label in the microsomes was irreversibly bound to the 54 kD cytochrome P-450 and 6% was irreversibly bound to the 52 kD cytochrome P-450. Since about 28% of the heme was irreversibly bound, the sum represents 61% of the total irreversibly bound heme-derived label in the microsomes. The immunoreactive 54 kD cytochrome P-450 found in the liver microsomes from CCl_4 treated rats was less than 80% of that present in liver microsomes of untreated rats, which suggests that the protein of the inactivated cytochrome P-450 maybe preferentially hydrolyzed in vivo.

Significance to Biomedical Research and Program of the Institute: Cytochromes P-450 are found in most tissues of the body. They exist in multiple forms with different specificities and are involved in the metabolism of steroids, prostaglandins, leucotrienes, fatty acids, drugs, and environmental chemicals. Inhibition of these enzymes, consequently, can have a profound effect on not only normal physiology but also the pharmacologic and toxicologic activities of xenobiotics.

Two general mechanisms for the irreversible inactivation of cytochrome P-450 by xenobiotics have been previously recognized. These processes have been termed 'suicidal' since they both involve inactivation by a metabolite produced by the

cytochrome P-450. In one case, the metabolite inactivates the enzyme by becoming bound to the heme prosthetic group whereas in the other case it binds covalently to the protein component of the enzyme. We have now found that there is a third mechanism for the irreversible inactivation of cytochrome P-450. In this case, the heme moiety itself is activated and binds irreversibly to the apoprotein portion of the enzyme. This process not only inactivates cytochrome P-450, but also appears to stimulate its catabolism. Although the mechanism of this reaction is not yet clearly understood, it is believed to be of general importance and promoted by factors that induce cellular lipid peroxidation.

Proposed Course of Project: We intend to do the following: 1) Determine the structure of the heme degradation products that are bound to apocytochrome P-450. This should help explain the mechanism of cytochrome P-450 destruction. It should also lead to a better understanding of the chemistry of the active site of cytochrome P-450. 2) Determine whether other xenobiotics that are known to inactivate cytochrome P-450 do so by this mechanism. This information may lead to the design of safer drugs that do not inactivate cytochrome P-450. In contrast, our studies may also be useful in designing specific 'suicide' inactivators of cytochrome P-450, which can be used as tools to investigate the functions of these enzymes. 3) Identify the catabolizing enzymes that degrade cytochromes P-450 and study their mechanisms of action. These enzymes may have an important role in the regulation of the steady state levels of cytochrome P-450.

Publication:

Davies, H.W., Satoh, H., Schulick, R.D. and Pohl, L.R.: Immunochemical identification of an irreveribly bound heme-derived adduct to cytochrome P-450 following CCl_4 treatment of rats. Biochem. Pharmacol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00971-02 LCP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Decreases in cytochrome P-450 caused by tunicamycin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Yogendra Singh	Visiting Fellow	LCP	NHLBI
Other Investigators:			
C.T. Liu	Chemist	LCP	NHLBI
V. Shirhatti	Vist.Fellow	LCP	NHLBI
G. Krishna	Section Chief	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Tunicamycin-induced a concentration dependent decrease in cytochrome P-450 in rat hepatocyte with a maximum decrease of 44% by 1 ug/ml tunicamycin after 3 days. It also decreased NADPH-cytochrome c reductase to a lesser extent. The effect of tunicamycin on these two systems were not evident till after 24 hr of incubation. However, the inhibitory effect of tunicamycin on protein synthesis was evident as early as 2 hr and was maximum at 24 hr. When the proteins were separated by SDS PAGE, there was a marked reduction in cytochrome P-450 isozymes as well as marked reduction in the incorporation of [35S]-methionin in these isozymes by tunicamycin. Various homologs of tunicamycin also decreased both cytochrome P-450 and protein synthesis in hepatocytes. The effect of tunicamycin on protein synthesis as well as cytochrome P-450 was due to a reduction in the synthesis of mRNA was investigated by the effect of tunicamycin on (14C)-uridine incorporation into RNA and isolation of RNA followed by translation of RNA into cytochrome P-450. Tunicamycin reduced total RNA synthesis as well as a reduction in the synthesis of mRNA coding for cytochrome P-450. Tunicamycin did not have any effect on the replication of DNA even though it inhibited DNA repair.

Project Description:

Objectives: Tunicamycin an antiviral antibiotic has been shown to inhibit glycosylation of proteins by inhibiting condensation of dolichol phosphate and UDP N-acetyl glucosamine. We reported earlier that tunicamycin causes a marked decrease in liver cytochrome P-450 and metabolism of drugs. We have investigated the mechanism by which tunicamycin decreases cytochrome P-450 in liver by studying its effect on rat liver cells maintained in culture.

Methods Employed: Hepatocytes were isolated by collagenase prfusion of liver from male Sprague Dawley rats weighing 200 g. The cells were cultured in Williams E medium on collagen coate plates. Tunicamycin was added to the medium and the cells were incubated under 95% air:5% CO_2. Medium was replaced every 24 hr. Cells were solubilized in emulgen buffer for estimation of cytochrome P-450 and NADPH-cytochrome c reductase. The isozyme pattern was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).

Protein, RNA and DNA synthesis in the cells were investigated by incorporation of [^{35}S]-methionine, [^{14}C]-uridine and [^{3}H]-thymidine respectively into TCA precipitable fraction from the cell.

Effect of tunicamycin on synthesis of cytochrome P-450 mRNA was investigated by isolation of RNA from the cells followed by cell free translation using rabbit reticulocyte lysate and [^{35}S]-methionine containing amino acid mixture. The translated [^{35}S]-labeled protein were separated by SDS-PAGE followed by autoradiography.

Major Findings: Tunicamycin-induced a concentration dependent decrease in cytochrome P-450 in rat heaptocyte with a maximum decrease of 44% by 1 µg/ml tunicamycin after 3 days. It also decreased NADPH-cytochrome c reductase to a lesser extent. The effect of tunicamycin on these two systems were not evident till after 24 hr of incubation. However, the inhibitory effect of tunicamycin on protein synthesis was evident as early as 2 hr and was maximum at 24 hr. When the proteins were separated by SDS PAGE, there was a marked reduction in cytochrome P-450 isozymes as well as marked reduction in the incorporation of [^{35}S]methionine in these isozymes by tunicamycin. Various homologs of tunicamycin also decreased both cytochrome P-450 and protein synthesis in hepatocytes. The effect of tunicamycin on protein synthesis as well as cytochrome P-450 was due to a reduction in the synthesis of mRNA was investigated by the effect of tunicamycin on (^{14}C)-uridine incorporation into RNA and isolation of RNA followed by translation of RNA into cytochrome P-450. Tunicamycin reduced total RNA synthesis as well as a reduction in the synthesis of mRNA coding for cytochrome P-450. Tunicamycin did not have any effect on the replication of DNA even though it inhibited DNA repair.

Significance to Biomedical Research to Program of the Institute: The finding that tunicamycin decreases cytochrome P-450 in hepatocytes by specific inhibition of synthesis of mRNA coding for cytochrome P-450 may explain the mechanism of tunicamycin induced decrease in cytochrome P-450 *in vivo*. Tunicamycin has been used as a tool for the study of glycosylation of proteins on the assumption that

it has no effect on protein or RNA synthesis. This assumption may have to be modified in view of present findings. In the interpretation of the results obtained with tunicamycin, the possible effects of m-RNA and protein synthesis may also have to be considered in addition to its effect on protein glycosylation.

Proposed Course of Project: Since the effect of tunicamycin on cytochrome P-450 has been clarified, these studies will be terminated.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 00972-02 LCP

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms of inactivation of biogenic amines by microvascular endothelial cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Michael A. Beaven	Deputy Chief	LCP	NHLBI
Other:	Audrey Robinson-White	Guest Researcher	LCP	NHLBI

COOPERATING UNITS *(if any)*

Dr. Stephen Baylin, Johns Hopkins School of Medicine, Baltimore; Dr. Thomas Olivecrona, Dept. of Chemistry, Univ. of Umea, Sweden.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Microvascular endothelial cells from rat and guinea pig fat pad bound the histamine-metabolizing enzyme diamine oxidase (DAO). The extent of binding was dependent on concentration of enzyme activity and tissue. Saturation of binding sites with 5000 units DAO/ml resulted in levels of bound activity (up to 11-13 units/mg endothelial cells) in excess of that observed in all tissues except placenta. Scatchard plots suggested that there were at least two DAO binding sites (apparent K_m 9 and 250 nM). Although the same cell preparations bound lipoprotein lipase (LPL) the presence of LPL did not interfere with binding of DAO activity except when cells were exposed to high concentrations of LPL. Alternatively, bound DAO activity was partially displaced (up to 33%) only with high concentrations (30 µg/ml) of LPL. DAO activity may thus be bound to at least two populations of sites, one of which may bind LPL. Both enzymes, however, were displaced by heparin (0.05 to 5 units/ml) and their binding was impaired by prior treatment of cells with glycosoaminoglycan degrading enzymes. The demonstration of DAO binding to vascular endothelial cells provides a further example of the ability of these cells to bind enzymes at their surface and thereby to act on biologically active substances in the circulation.

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Project Description:

Objectives: This project is concerned with the mechanism of clearance of histamine from the circulation after physiological or immunological stimuli. Labeled histamine is rapidly removed from the circulation (Klin. Wochenchr 60: 873, 1982; Brit. J. Pharmacol. 49:569,1973) and appears as methylated and deaminated metabolites in all tissues within minutes of injection. When infused through whole animals or isolated organs, histamine is cleared upon a single passage through different vascular beds. We have shown (see previous reports Z01 HL 00631-01 to 03 LCP) that microvascular endothelial cells contain high levels of the histamine degrading enzyme histamine methyltransferase (HMT), and diamine oxidase (DAO). Endothelial cells may thus be a major site of inactivation of circulating histamine.

In this final report of this series we show that HMT activity is present in the endothelial cell cytosol whereas DAO activity is highly localized on the surface of the plasma membrane. We have defined the characteristics of DAO binding with both crude and highly purified DAO preparation from rat placental extracts. Comparisons have been made with lipoprotein lipase (LPL) binding to endothelial cells as both LPL and DAO are released into the circulation within seconds of administration of heparin (Baylin, Beaven et al., J.Clin.Invest. 52:1985, 1973). LPL has been shown by others to bind to cultured endothelial cells and be displaced from these cells from sites believed to contain heparan sulfate-like moieties.

Methods Employed: 1) Isolated cell preparations and assay procedures: Microvascular andothelial (MVE) cells from rat and guinea tissues were prepared and their viability was assessed as described in project reports for 1980 to 1983. They were assayed for HMT and DAO activities by procedures outlined in the same reports. I^{125} LPL was assayed by gamma counting of I^{125} content. Sonified cell extracts were also centrifuged (10,000 g x 30 min) to permit assay of enzyme activities in membrane and supernatant fractions.

2) Enzyme preparations and extracts. DAO soluble extracts were prepared from rat or human placental extracts and purified by use of sepharose cadaverine affinity columns. LPL and I^{125} LPL were isolated from bovine milk by heparin - sepharose columns (Biochem.J.167,109,1977;B.B.A. 397,294, 1975).

3) DAO and LPL binding studies. These were performed as described in detail in last year's report. Sensitivity of DAO and LPL binding sites to protelytic enzymes was tested by incubating fat pad MVE cells with trypsin, pronase or heparinase before measurement of binding activity. Times of incubations and enzyme concentrations were varied as indicated.

Major Findings: 1) Intracellular distribution of DAO and HMT activities. All of the HMT activity was recovered in the cytosolic fraction of MVE cell preparations from guinea pig fat pad, brain and cerebral cortex. Little or no HMT activity was present in MVE cell preparations from the same tissues in rat. DAO activity, which was present in all MVE preparations tested from rat and guinea pig, was recovered largely (>70%) from the membrane fraction.

2. DAO binding: preliminary studies with placental extracts. Both the rat and guinea pig microvascular cell preparations bound DAO activity present in rat and human placental preparations. The percentage of DAO activity bound ranged from 16-28% in rat MVE cells and up to 44% with guinea pig MVE cells. Binding was apparent at 4° and 37° and at both temperatures equilibrium was reached within 5 to 15 min. Subsequently, there was some decrease in the amount of bound activity at 37° not at 4°. Binding was observed with microvessel preparations obtained from fat pad of two strains of rat as well as guinea pig. The enzyme activity bound was related to concentration of enzyme activity and amount of tissue. Binding did not appear to interfere with the catalytic activity of enzyme; DAO activity measured in intact cells was not significantly different from that in sonicated rat MVE cell suspensions.

3. Displacement of DAO activity by heparin. With cell preparations previously treated with placental extracts, bound DAO activity was released rapidly into the medium by heparin. Release was observed with as little as 0.2-0.4 µg heparin/ml and near maximal release with 8 µg heparin/ml.

4. Kinetics of binding of DAO and LPL activity: Studies with purified enzyme prepartions. Rat placental DAO activity was purified to near heterogeneity to yield a protein of 97,000 D which constituted approximately 85% of the total protein in the preparation. With rat fat pad MVE preparations, saturation of binding with the purified preparation was approached with levels of activity above 5000 units DAO activity/ml to yield cell pellets containing 11-13 units/mg tissue. Scatchard plot of the data was consistent with binding to at least two classes of sites to give apparent dissociation constants of 92 $\pm$ 51 units/ml (9 nM) and 2453 $\pm$ 175 units/ml (250 nM).

Studies with purified LPL and DAO proportions indicated that the amounts bound at low concentrations of LPL (1 µg/ml) or DAO (100 units or 1 µg DAO/ml) were similar (0.9-1.9 ng LPL and 0.5 - 1.0 ng DAO/µg cell protein). The presence of LPL on the endothelial cell did not affect binding of DAO activity except when cells were exposed previously to high (35 µg LPL/ml or greater) concentrations of LPL and DAO was displaced to only a limited extent (33%, $p < 0.05$) by high concentrations (30 µg/ml) of LPL. Additionally significant displacement of bound LPL was observed with only high concentrations (5,000 units/ml) of DAO activity.

5) Sensitivity of DAO and LPL binding sites to enzymatic degradation. Following incubation (5 min) of cells with 6,12,60 and 120 m/U/ml heparinase, DAO binding was reduced by, respectively, 26 $\pm$ 4, 55 $\pm$ 3, 59 $\pm$ 3 and 68 $\pm$ 3%. Similar treatment with Pronase (1 PUK unit/ml) or trypsin (0.05%) resulted in 57 $\pm$ 8 and 60 $\pm$ 7% loss of binding activity. Treatment with trypsin (0.05% for 3 min) was also shown to reduce DAO and LPL binding by, respectivley, 53 and 47%. None of these treamtents caused loss of cell viability. These data suggested that DAO (and LPL) were bound to superficial sites on the cell surface.

Significance to Biomedical Research and Program of the Institute. The presence of high DAO activity on the surface and high HMT activity in the cytosol of endothelial cells may account for the rapid clearance of histamine across vascular beds. As only small increases in plasma histamine (>5 ng/ml) are associated with cardiovascular reactions and modest increases (10-20 ng/ml) with severe anaphylatic reactions or death in man, the presence of such enzymes in endothelial cells may play a critical role in minimizing these reactions.

Proposed Course of Project: The project is terminated.

Publications: Robinson-White, A., Baylin, S.B., Olivecroma, T. and Beaven, M.A.: Binding of diamine oxidase activity to rat and guinea pig microvascular endothelial cells: Comparisons with lipoprotein lipase binding. J. Clin. Invest., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00973-01 LCP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical mechanisms of mast cell degranulation: Potentiating pathways

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Michael A. Beaven	Deputy Chief	LCP	NHLBI
Other Investigators:			
Elizabeth WoldeMussie	Staff Fellow	LCP	NHLBI
Kazutaka Maeyama	Visiting Fellow	LCP	NHLBI
Denise Guthrie	Chemist	LCP	NHLBI

COOPERATING UNITS *(if any)*

Cooperating Unit: T.R. Hesketh and J.C. Metcalfe, Dept. Biochemisty, Univ. of Cambridge, Cambridge, England

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	0.6	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies with the calcium ionophore, A23187, and the phorbol ester, TPA, in RBL-2H3 cells indicate that increases in cytosolic calcium concentrations or activation of kinase C alone is an inadequate stimulatory signal for histamine release. A combination of the signals provide a stimulus for release. Exposure of cells, for example, to low concentrations of A23187 and TPA, which by themselves do not stimulate release, can induce near maximal histamine secretion. However, activation of kinase C may serve both to promote secretion and to inhibit initial stimulatory signals. In antigen stimulated cells, TPA at 5-50 nM concentrations markedly suppresses phosphoinositide breakdown and increases cytosolic calcium concentrations in a concentration-dependent fashion. Later stages of the secretory process appear to rely on Ca2+-calmodulin dependent steps. Calmodulin inhibitors at pharmacologically relevant concentrations inhibit histamine release. Inhibition of phosphoinositide breakdown is observed with some of these inhibitors but only at high concentrations of drug (50-100 nM).

Project Description:

Objectives: Many substances that activate calcium-dependent responses also stimulate breakdown of membrane phosphoinositides, namely phosphatidyl-inositol (PI), phosphatidylinositol, 4-phosphate (PIP) and phosphatidyl 4,5-bisphosphate (PIP_2) to release, respectivley, inositol 1 monophosphate (IP), inositol 1,4-bisphosphate (IP_2) and inositol 1,4,5-triphosphate (IP_3) into the cell cytosol. PI is rapidly converted to PIP and PIP_2 by membrane bound kinases in the presence of ATP (see also project report No. Z01 HL 00937-01 LCP). Cleavage of the phosphoinositides is catalyzed by the enzyme(s) phospholipase C. In some cell systems a PIP_2-specific phospholipase C has been identified in the cell cytosol which becomes attached to the cell membrane in stimulated cells. In these systems, cell activation leads to formation of IP_3 which is then sequentially degraded to IP_2, IP and inositol by the action of phosphatases. IP_3, at concentrations produced within the cell (1-10 uM), has been shown to release up to 30% of the Ca^{2+} bound to endoplasmic reticulum. Hence the proposal that IP_3 acts as a second messenger to recruit internal Ca^{2+} ions (Nature 309:63 and 562, 1984). It is clear, however, from our work in 2H3 cells and smooth muscle cells (J.Biol.Chem. 259:7129 and 7137, 1984 and in press) that calcium signals can be generated exclusively by Ca^{2+} influx (e.g. antigenic stimulation of 2H3 cells) or through mobilization of internal Ca^{2+} stores (e.g. stimulation of smooth muscle cells with 100 nM angiotensin II). Although it is probable that PI breakdown is required for Ca^{2+} influx in 2H3 cells the exact mechanism remains unclear. Our data do indicate that PI breakdown may activate multiple synergistic pathways and that IP rather than IP_3 may be the principal inositol phosphate generated (see related report No. Z01 HL 00972-02 LCP).

The second cleavage product of all 3 phosphoinositides, diacylglycerol (DAG), has also been shown to activate protein kinase C - an event mimicked by the phorbol ester TPA - to promote alternate stimulatory pathways in calcium dependent secretory cell systems (Advances in cyclic nucleotide and protein phosphorylation research 18:119, 1984).

This project was initiated to examine for possible alternate stimulatory pathways in 2H3 cells. The project has allowed us to investigate at a biochemical level some of the phenomena described in related reports.

Methods Employed: All procedures were performed as described in the reports and publications cited above. In general simultaneous measurements of increases in cytosolic calcium concentrations ($[Ca_i^{2+}]$) and PI breakdown were made with cells in suspension culture. PI breakdown and histamine release were compared in separate (but identical) cluster plate cultures of 2H3 cells (see J. Biol. Chem. 259:7137, 1984). Various test agents were added 10 min before addition of stimulant. In some experiments, test agents were added after addition of stimulant where indicated. Phospholipase C was assayed by the procedure of Lapetina (BBA 752:329,1983).

Major Findings: 1) Response of 2H3 cells to Ca ionophore, A23187. The ionophore stimulated histamine release only at concentrations of 200 nM or greater. Release was related to concentration of ionophore. This release ranged from 5 ± 2% with 200 nM A23187 to 59 ± 3% with 500 nM A 23187. However, significant increases in $[Ca_i^{2+}]$ were apparent with as little as 12.5 nM A23187 (e.g. 100 to 178 nM $[Ca_i^{2+}]$). Although the responses were variable from experiment to experiment, the maximal increases in $[Ca_i^{2+}]$ ranged from 150 to 370 nM with 50 nM A23187 and 360 to 700 nM with 100 nM A23187. Concentrations of 200 nM produced increases > 1,500 nM. At none of these concentrations, was significant breakdown of PI observed (< 0.5%).

2) Responses to antigen or oligomeric IgE preparations. As described in related reports, both PI breakdown and increases in $[Ca_i^{2+}]$ were dependent on concentration of antigen (in cells primed with antigen-specific IgE) or IgE oligomers. Even with low concentrations of stimulant (e.g. 5 ng/ml oligomer) and occupancy of <0.05% of the IgE receptors, measurable PI breakdown (1.5%), increases in $[Ca_i^{2+}]$ (250 nM) and histamine release (10-15%) was observed. With optimal concentrations of stimulant, maximal increases in $[Ca_i^{2+}]$ ranged from 1,200 nM (with ovalbumin, 10 µg/ml) to 1,600 nM (with IgE oligomers, 1µg/ml) and PI breakdown ranged from 30-70%. Histamine release varied from 45 to 65%.

The above data indicated that increases in $[Ca_i^{2+}]$ were always associated with histamine release when cells were stimulated with antigen or oligomer but not when stimulated with A23187. A striking example of this disparity was evident in one experiment where both ovalbumin (0.05 ng/ml) and A23187 (50 nM) elicited identical increases in $[Ca_i^{2+}]$ (350 nM). Histamine was released (17 ± 2%) in response to antigen but not in response to ionophore (<1%).

3) Activation of protein kinase C with TPA. The phorbol ester alone (5-100 nm) had no effect on 2H3 cells. TPA markedly potentiated histamine release induced by A23187. This potentiation was most apparent with concentrations of A23187 which by themselves (e.g. 100-200 nM) induced little histamine release. Maximal effects were observed with 20-30 nM TPA but as little as 5 nM TPA enhanced histamine release 4-fold (with 200 nM A23187).

In contrast, TPA did not significantly enhance histamine release induced by low or high concentrations of antigen. It did, however, markedly depress PI breakdown and the Ca signal at concentrations of 5-100 nM in a concentration-dependent fashion. In fact conditions could be manipulated (0.05 µg/ml ovalbumin, 50 n M TPA) so that PI breakdown and Ca signal were virtually abolished while histamine release (10 ± 2%) was still maintained. Although 2H3 cells possessed high phospholipase C activity (55 pmoles/sec/10^6 cells with 0.5 mM PI), TPA did not suppress this activity. With extracts of unstimulated cells, phospholipase C activity was expressed only in the presence of Ca^{2+} ions.

In all the above experiments, removal of external Ca^{2+} or blockade of Ca^{2+} influx with La^{3+} abolished all responses whether TPA was present or not.

Effect of calmodulin inhibitors. These studies are incomplete but the data are sufficient to indicate that these inhibitors suppress histamine release at pharmacologically relevant concentrations. The order of potency is Calmidazolium > TFP > W5> W7. Inhibition of PI breakdown is observed with some of them but only at high drug concentrations (50-100μM). To date, no dramatic alteration of Ca signal has been observed.

Significance to Biomedical Resarch: The above studies suggest the following: 1) an increase in cytosolic Ca^{2+} concentration by itself is an inadequate stimulatory signal; 2) increases above 1,500 nM Ca^{2+} may, however, initiate secretion but this secretion could result from direct activation of phospholipase C by Ca^{2+} (and work in progress); 3) in the presence of PI breakdown (e.g. antigen stimulation) increases in $[Ca_i^{2+}]$ are associated with histamine release but activation of kinase C alone (e.g. TPA alone) is an insufficient stimulus for release: 4) kinase C activation leads to suppression of PI breakdown and Ca^{2+} signal, a phenomon that could indicate a feed-back inhibitory loop; 5) Ca^{2+}-calmodulin dependent step(s) appear to be involved at later stages of the secretory process and finally 6) Ca^{2+} is a critical requirement for initiation (e.g. PI breakdown) and subsequent steps in the signal cascade. Although we suspect that further work will reveal additional elements, the data begin to provide a working model from which useful experimental approaches can be predicted.

Proposed Course of Project: The above analysis is based on the assumption that TPA acts through activation of kinase C. Further work will include characterization of kinase C and phospholipase C in 2H3 cells, the effects of TPA, and DAG on these enzyme systems and examination (with Dr. Robert Adelstein) of the pattern of protein phosphorylation in stimulated 2H3 cells.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00974-01 LCP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

ATP dependency of signal generation and secretion in rat basophil leukemic cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Theresa N. Lo	Research Chemist	LCP	NHLBI
Others:	Michael A. Beaven	Deputy Chief	LCP	NHLBI
	Wilford Saul	Chemist	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md.

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	0.8	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

On antigenic stimulation, rat basophil leukemic (RBL-2H3) cells respond with rapid increases in free cytosol calcium concentration, hydrolysis of PI (phosphatidylinositol and its phosphorylated derivatives), and histamine release. These processes depend on the presence of ATP (J.Biol.Chem. 299:7129, 7137, 1984). In the present studies, complete depletion of cellular ATP in 2H3 cells could be achieved only when the cells were treated with antimycin A (0.1 μM) in a glucose-free medium. Under these conditions, cellular ATP content was <1% of the normal level (0.05 ± 0.05 as compared to 5.8 ± 0.06 ug/million in untreated cells) and the release of inositol phosphates (i.e., PI breakdown) and of histamine from the cells were 7% and 13% of the maximum, respectively. The suppression of PI breakdown and histamine release by antimycin A was dose-dependent and were highly correlated (correlation coefficient = 0.99, $P < 0.001$). The ATP-dependent nature of the two processes suggest that a critical phosphorylation step is required for the initiation of PI breakdown and consequently histamine release.

Project Description:

Objectives: The ATP, calcium-dependent release of histamine and other inflammatory mediators from tissue mast cells and blood basophilis is the primary event in a variety of acute allergic and inflammatory conditions. The release can be initiated by interaction of antigen with membrane-bound IgE molecules on the cell surface or by exposure of the cells to a variety of stimulatory agents such as calcium ionophores and compound 48/80. Recently, Beaven et al., (J.Biol.Chem.299:7129, 7137,1984) reported that phosphatidylinositol (PI) and its phosphorylated derivatives (PIP, PIP_2) were rapidly broken down in aggregated ovalbumin-stimulated rat basophil leukemic (2H3) cells with a time course that coincides with the generation of the Ca signal. Furthermore, the rate of histamine release was maximal during the period when cytosolic calcium concentration ($[Ca^{++}]i$) was increasing rapidly. Maintenance of the Ca signal was dependent on the supply of ATP as the signal could be ablated by addition of metabolic inhibitors (10 mM deoxyglucose plus 10 mM sodium azide). Although ATP levels were reduced by 96-98%, the cells were still capable of maintaining low intracellular calcium levels, and were presumably viable. However, the almost complete depletion of ATP did not allow inferences to be drawn as to the role of ATP in signal transduction mechanisms. We have extended these studies by more refined control ATP levels in the RBL-2H3 cells.

Methods Employed: Priming of RBL-2H3 cells with IgE, labeling of cells with [^{3}H] inositol, and measurement of histamine release, PI breakdown, and the cytosolic calcium concentration (measured by the quin 2 probe) were done as described elsewhere (J.Biol.Chem. 299:7129,7137,1984). In the studies described here RBL-2H3 cells were plated in 2 identical cluster well plates (24 wells, $2x10^5$ cells/ well) and then incubated overnight. The plates were washed and treated exactly as described in report No. Z01 HL 00837-03 LCP. One plate was used to measure histamine and/or ATP content. The other was used to monitor release of inositol phosphates. Labeled phospholipids were assayed by TLC after extraction into chloroform.

Measurement of cellular ATP: ATP was assayed in a DuPont 760 luminescence biometer by measuring the intensity of light emitted during the ATP-dependent, luciferase catalyzed oxidation of luciferin. To each well was added 10 ul of either vehicle or antimycin A (final concentration 1-100 nM). The plates were placed in a 37°C water bath. Ten minutes later glutaldehyde - aggregated ovalbumin (10 ug/ml final) was added and incubation allowed to proceed for another 30 min. The reaction was terminated by addition of perchloric acid and the mixture was neutralized with 0.5 M KOH in 0.1 M morpholinoproprane sulfonic acid. With the described protocols, 10 ul from the diluted supernatant sample was sufficient for ATP measurement. ATP standards (0.01-1 ug/ml were prepared and assayed under similar conditions

Expression of results. Results are expressed either as a mean ± S.E. of at least 6 cultures or as a percentage of maximum release (i.e., release in the absence of antimycin A).

Major Findings: Dose-dependent reduction of ATP levels of RBL-2H3 cells treated with antimycin A. In the absence of glucose a 10 min incubation of the cells with antimycin A resulted in a dose-dependent decrease in cellular ATP content (column 2).

Antimycin [nM]	ATP content ($ug/10^6$ cells)	Inositol phosphate released (dpn/200,000 cells)	histamine released (% of maximum)
0	5.8 ± 0.6	3833 ± 97	100
1.0	4.9 ± 0.8	3563 ± 133	85 ± 13
2.5	3.7 ± 0.9	1832 ± 199	71 ± 5
3.8	0.5 ± 0.2	532 ± 34	51 ± 1
5.0	0.5 ± 0.1	500 ± 33	23 ± 8
10.0	0.5 ± 0.03	266 ± 54	17 ± 6
100.0	0.05 ± 0.05	266 ± 67	13 ± 3

Inclusion of glucose (5.6 mM) in the incubation medium substantially decreased the effect of antimycin: ATP level returned to 85% of the normal level and PI breakdown remained essentially unimpaired. The results indicate that inhibition of both oxidative phosphorylation and glycolysis are required to totally suppress ATP production in these cells and that PI metabolism is highly ATP dependent (see also below).

Effect of ATP depletion on PI breakdown and histamine release. The decreases in ATP content observed in antimycin A-treated cells were accompanied by similar decreases in rates of PI breakdown (as indicated by the decreased release of inositol phosphates) and histamine release from the cells (columns 3 and 4, respectively). The suppression of PI breakdown and histamine release in antimycin A treated cells was highly correlated with a correlation coefficient of 0.99 ($P<0.001$).

Of the total [^{3}H] inositol incorporated into the cells 96-98% of the label was incorporated into phosphatidylinositol (PI) with the remaining 2-4% distributed between phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-diphosphate (PIP_2).

Significance to Biomedical Research and the Program of the Institute. Based on indirect evidence, polymerization of tubulin to microtubules has been proposed to be one mechanism for extrusion of mast cell granules. Involvement of other cytoskeletal elements such as the microfilaments (actin-myosin) cannot be ruled out. The explosive extrusion of granules during degranulation has lead some to think that it is an energy-dependent process. However, published data do not differentiate between the ATP-dependency of signal cascade mechanism(s) from that of secretion. Our studies suggest that a phosphorylation step is critical for PI breakdown and consequently histamine release.

Proposed Course of Project: Future studies include correlation between the decreased PI breakdown and Ca^{++} signal resulting from ATP depletion. In addition, the influence of Ca^{++} fluxes (influx and efflux) and cellular ATP content on the hydrolysis of the phosphoinositides and the polyphosphoinosities and phosphorylation of phosphatidylinositol will be examined.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00975-01 LCP

PERIOD COVERED: October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Signal cascade mechanisms in histamine releasing and nonreleasing RBL clones

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Elizabeth WoldeMussie

Other Investigators:

Michael A. Beaven	Deputy Chief	LCP	NHLBI
Kazutaka Maeyama	Visiting Fellow	LCP	NHLBI

COOPERATING UNITS *(if any)*: Dr. Reuben Sirganian, National Institute of Dental Research

LAB/BRANCH: Laboratory of Chemical Pharmacology

SECTION: Cellular Pharmacology

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Antigen stimulation of rat basophilic leukemia (2H3) cells is associated with increase in intracellular calcium (Ca signal), substantial phosphatidyl inositol (PI) breakdown as well as histamine release. Different clones of the RBL cells, however, showed varied responses to antigen from none at all to about 80% of the responses observed in 2H3 cells. In partially defective clones, PI breakdown and calcium signal were more transient than was observed in 2H3 cells. In all but one of these clones the Ca signal and PI breakdown were correlated. The exception was a clone in which PI breakdown and histamine release but not calcium signal were observed. In 2H3 cells PI breakdown the Ca signal and histamine release were highly temperature dependent. There was no response below 20°C and a maximal response at 36-37°. At temperatures above 37° the responses were suppressed and by 40-42° they were completely inhibited. The effects of temperature were reversible. The findings confirm previous data that although PI breakdown and calcium signal are in general highly correlated, PI breakdown can occur independently of calcium signal.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives: Histamine release from mast cells is a critical event in many inflammatory reactions. It is an energy and calcium requiring process. Last year we reported several studies on the mechanism of histamine release in relation to the metabolism of the membrane phosphoinositides (PI) and generation of calcium signal. During the past year used several clones of the rat basophilic leukemia cell line that show varied reponses to stimulation. The objectives are to determine whether the Ca signal and PI breakdown are always associated events and to identify specific defects in the PI, kinase C, Ca^{2+} signal mechanisms. We have also studied the effects of temperature on the signal generation and histamine release.

Methods Employed: 1) Cell culture - The rat basophilic leukemia (RBL) clones were cultured and maintained in supplemented minimum essential medium in Earle's balanced salt solution (JBC 259:7129-7136,1984). The RBL clones 1A3, 1D2, 1B3 and 2B1 were cultured in the above medium containing 10^{4}M bromodeoxyuridine (BUDR) and clones 1D5,2B6,1B3 and 1D3 were maintained in medium containing 10^{-4}M thioguanine (TGR). The cells were either BUDR® or TG resistant and thus designated BUDR® or TG®.

2) Experimental Procedures. Measurement of intracellular calcium (Ca) in cell suspensions were undertaken with the fluorescent probe quin 2 as described in JBC 259:7129-7136,1984. Phosphatidyl inositol (PI) breakdown was measured in cultures in cluster plates that were previously labeled with [^{3}H]-inositol by methods described in JBC 259:7137-7142,1984. In both procedures cells were passively sensitized with monoclonal IgE specific for ovalbumin. Degranulation was induced by adding aggregated ovalbumin. All clones took up and hydrolyzed quin 2 ester as demonstrated by uptake of [^{3}H] labeled quin 2 ester and shift in emission spectra as described previously.

3) Effect of temperature on degranulation process. RBL 2H3 cells were incubated at different temperatures (15-42°C) and the changes in Ca signal, PI breakdown and histamine release were measured after stimulation with ovalbumin.

Histamine was determined by radioenzymatic assay (JPET 224:620-626, 1983).

Major Findings: As shown in a previous report (Z01 HL 00937-02 LCP) the response of 2H3 cells to antigen (ovalbumin) stimulation resulted in an increase in intracellular Ca (from 0.1 to 1.0 uM), PI breakdown of upto 60% and histamine release of upto 50-60%. 1A3 cells, however, failed to exhibit any of the above responses although these cells were able to bind IgE. Amoung the clones that have since been tested BUDR® 1B3 and BUDR® 2B1 were totally unresponsive to antigenic stimulation. The other clones TG 1D5, 2B3, 1D3, and BUDR® 1D2 had responses, ranging from 9 to 86% of those of 2H3 cells. The absence or deficiency in PI breakdown was not due to defects in PI synthesis. All clones, responsive and nonresponsive took up [^{3}H]-myoinositol and incorporated the labeled sugar into the phosphoinositide pools to approximately the same extent.

The time course of PI breakdown showed that the initial rates of breakdown in the responsive clones were similar. However, in these clones PI breakdown ceased by 10 min whereas in 2H3 cells the response persisted for more than 30 min. Similarly the initial rate of increase in Ca signal was the same for the partially defective clones and 2H3 cells but the extent of increase was attenuated to varying degrees in the clones as compared to 2H3 cells. In general there was a close correlation between Ca signal and PI breakdown. However, the increase in Ca signal in BUDR® 1D2 was larger than expected from the extent of PI breakdown (10%) and the opposite was the case for TGR® 1B3 which showed very small changes in Ca signal (< 50 nM) compared to the amount of histamine released (30% net) and extent of PI breakdown (10%).

There was appreciable amount of phospholipase C activity (the enzyme responsible for PI hydrolysis) in all cell clones even in those clones such as 1A3 that showed no response to antigen stimulation.

Effect of temperature on cellular response in 2H3 cells. The increase in intracellular Ca, PI breakdown and histamine release in antigen stimulated cells was found to be temperature dependent. There was no response below 20°C. All three responses increased in rate as well as extent as the temperature was raised from 20 to 38°C. At 39° or higher, however, all responses were markedly depressed with virtually no response at 40-42°C. This impairment of response was largely reversible (75-85%) when the incubation temperature was readjusted to 37°.

The stimulated increase in intracellular Ca concentration was maintained over a long period (30 min) at lower temperatures (30-34°C) but faded progressively as the temperature was increased. At 39°C the Ca signal was transient. The decrease in response at high temperature was not due to inactivation of phospholipase C because the enzyme activity at 40° was equvialent to that at 37°. A possible explanation was that changes in membrane fluidity may alter coupling of receptor aggregation events to phospholipase C activation.

Significance to Biomedical Research and Program of the Institute: The 2H3 cells have been instructive in studies of the mechanisms of secretory response. The availability of other RBL clones which are defective in their histamine releasing ability may provide additional models to study processes involved in cell secretion. Studies with temperature show that cellular PI/Ca signal mechanisms cease with slight increases in temperatures above 37°. This finding may have important clinical implications.

Proposed Course of Project: The correlation of PI breakdown and Ca signal will be investigated further. Enzymes involved in these processes will be studied especially in the defective RBL clones.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00976-01 LCP

PERIOD COVERED: October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Drug-induced peroxisomal proliferation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Y. Singh	Vist. Fellow	LCP	NHLBI
Others:			
V. Shirhatti	Vist. Fellow	LCP	NHLBI
G. Krishna	Chief, Section	LCP	NHLBI

COOPERATING UNITS *(if any)*: Dr. Dennis Feller, College of Pharmacy, Univ. of Ohio, Columbus, Ohio

LAB/BRANCH: Laboratory of Chemical Pharmacology

SECTION: Drug Tissue Interaction

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hepatocytes were isolated from livers of male Sprague Dawley rats weighing 250 g and treated with various hypolipidemic drugs. Peroxisomal proliferation was assessed by increase in peroxisomal enzymes carnitine acetyltransferase (CAT), carnitine palmitoyltransferase (CPT) and an 80K protein which was isolated by gel electrophoresis. Clofibric acid and ciprofibrate increased CAT activity about 2 fold in liver cells with smaller increase in 80K protein in 48-72 hr. Ciprofibrate was more potent than clofibric acid (EC50 for ciprofibrate = 50 μM vs EC50 for clofibric acid = 300 μM). Maximal increase in CAT activity induced by these two drugs individually were not additive. Ciprofibrate induction of CAT was blocked by cycloheximide indicating de novo synthesis of enzyme. Elevations in 80K protein by these drugs were similar to the increases in CAT. Both drugs induced [35S]methionine incorporation into protein as well as into various peroxisomal enzyme. RNA-synthesis as estimated by incorporation of 3H uridine was measured by these drugs. Drug induced proliferation of peroxisomes were also confirmed by electron microscopic examination.

When hepatocytes were tested with clofibric acid, it increased mRNA synthesis coding for various peroxisomal marker enzymes such as catalase and CAT as demonstrated by translation of RNA in vitro using rabbit reticulocyte lysate and [35S]methionine containing amino acid mixture.

Project Description:

Objectives: Various hypolipidemic drugs such as clofibrate and ciprofibrate markedly induce peroxisomal proliferation in rat liver. However, the drug induced peroxisomal proliferation is not clear. The main objective of this study has been to develop a simple in vitro model for the drug induced peroxisomal proliferation and to investigate the mechanisn of induction at molecular level.

Methods Employed: Hepatocytes were isolated from male Sprague Dawley rats weighing 250 g by the collagenase perfusion method. Cells were cultured in Williams E medium on collagen coated plates. Clofibric acid and ciprofibrate were added to the medium and was incubated under 95% air and 5% CO_2. Medium was replaced every 24 hr. Cells were solubilized in Emulgen buffer for estimation of carnitine acetyltransferase, and carnitine palmitoyltransferase.

Protein and RNA synthesis in the cells were investigated by incorporation of [^{35}S]-methionine and [^{3}H] uridine into TCA precipitable materials from the cells.

Effect on clofibric acid on synthesis of carnitine acetyltransferase mRNA was investigated by isolation of RNA from clofibric acid treated cells. RNA was translated in vitro using rabbit reticulocytes lysate and [^{35}S]-methionine containing amino acid mixture. Proteins were separated on SDS-PAGE followed by autoradiography.

Major Findings: Peroxisomal proliferation was assessed by increase in peroxisomal enzymaes carnitine acetyltransferase (CAT), carnitine palmitoyltransferase (CPT) and an 80K protein which was isolated by gel electrophoreses. Clofibric acid and ciprofibrate increased CAT activity about 2 fold in liver cells with smaller increase in 80K protein in 48-72 hr. Ciprofibrate was more potent than clofibric acid (EC_{50} for ciprofibrate = 50 μM vs EC_{50} for clofbric acid = 300 uM). Maximal increase in CAT activity induced by these two drugs individually were not additive Ciprofibrate induction of CAT was blocked by cycloheximide indicating de novo syntheses of enzyme. Elevations in 80K protein by these drugs were similar to the increase in CAT. Both drug induced [^{35}S]-methionine incorporation into protei as well as into various peroxisomal enzyme. RNA-synthesis was increased by these drugs. Drug induced proliferation of peroxisomes were also confirmed by electron microscopic examination.

When hepatocytes were treated with clofibric acid, it increased mRNA synthesis coding for various peroxisomal enzyme marker such as catalase and CAT as demonstrated by translation of RNA in vitro using rabbit reticulocyte lysate and [^{35}S]-methionine containing amino acid mixture. [^{35}S] proteins were isolated by gel electrophoresis and visualized by autoradiography.

Significance to Biomedical Research and to Program of the Institute: The finding that peroxisomal proliferation can be studied using liver cell in vitro will greatly help in elucidating the mechanism involved in drug induced proliferation of peroxisomes as well as various enzymes in the liver. It has been postulated that peroxisomal proliferation of rat and mice may lead to hepatocarcinoma. Our model may be useful in studying possible mechanisms by which cell transformation might result from peroxisomal proliferation.

Proposed Course of Project: We propose to isolate and purify various mRNA coding for various peroxisomal markers and utilize them for preparing cDNA probe. These probes will be used for assessing the increase in mRNA both at the nuclear as well as cytoplasmic level by various drugs.

Publication: Feller, D.R., Singh, Y., Shirhatti, V., Liu, C.T. and Krishna, G.: Cultured rat hepatocytes: In: In Vitro Toxicology, Vol.3, (A. Goldberg, Ed.), Liebert Publishers, Inc., NY 497-514 (1985).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00977-01 LCP

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Mechanism of anthracycline induced cardiotoxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: James B. Reese	Guest Researcher	LCP	NHLBI
Others:			
Vilas Shirhatti	Visiting Fellow	LCP	NHLBI
Gopal Krishna	Chief, Section	LCP	NHLBI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Chemical Pharmacology

SECTION: Drug Tissue Interaction

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.54	0.54	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Adriamycin and daunomycin caused a dose-dependent decrease in the uptake of radiolabeled adenine into cardiac myocytes in culture. These drugs inhibited adenine uptake at concentrations that result in no release of lactic acid dehydrogenase. Preincubation of the cells with daunomycin did not increase the inhibition of uptake. Moreover, daunomycin did not decrease the proportion of label incorporated into ATP-ADP or 5'-AMP even though it decreased the uptake and incorporation of adenine. Daunomycin did not alter the capacity of myocytes to synthesize 5'-AMP from adenine and PRPP when tested in cell extracts. Thus daunomycin did not have any effect on the conversion of adenine into nucleotides but had a major effect on the uptake mechanism. Daunomycin also inhibited the uptake of deoxyglucose and amino acids into cardiac myocytes at concentrations which had no effect on enzyme release. These results indicate that one of the early effects of anthracycline antitumor agents may be on the uptake of nutrients by cardiac cells. The inhibition of uptake of nutrients ultimately contributes to cell death.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives: The main objective of this study is to elucidate the mechanisms responsible for the cardiotoxicity of the anthracycline anti-tumor drugs of which adriamycin is the archetype. The major side effect of this class of agents is to induce cardiomyopathy resulting in cardiac failure which, once developed, is irreversible and fatal. We have previously utilized cultured neonatal rat cardiac myocytes as a model system to study the toxicity of this family of drugs. This group of drugs induced a dose-dependent cardiotoxicity which included loss of contractile activity, leakage of cellular enzymes such as lactate dehydrogenase and creatine kinase, and leakage of GSH, GSSG, and adenine nucleotides. In this study we have investigated the effects of adriamycin and daunomycin on the uptake of radiolabeled adenine into cardiac myocytes and compared this effect with the other toxicity induced by these drugs.

Methods Employed: The methods involved in the isolation and culture of neonatal rat cardiac myocytes have been described in previous reports. The cells were incubated with either [^{3}H] or [^{14}C] adenine and the uptake and incorporation into nucleotides were monitored by both the rate of disappearance of the label from the medium and by the appearance of the label inside the cell. Adenine nucleotides were extracted from the cells and separated by HPLC or by anion exchange chromatography columns. The cardiac myocytes were incubated with various concentrations of adriamycin and daunomycin for various time intervals in order to monitor the effects on adenine uptake and incorporation as well as to monitor enzyme release. The cells were also incubated with [^{35}S] methionine and [^{14}C] deoxyglucose to investigate the effects of the drugs on amino acid as well as glucose uptake. Triton-X 100 solubilized cell extracts were incubated with [^{14}C] adenine and phosphoribosyl pyrophosphate (PRPP) in order to measure 5'-AMP synthesis by cell extracts.

Major Findings: Both [^{14}C] and [^{3}H] adenine were taken up by the cardiac myocytes at identical rates and were incorporated into nucleotides. Most of the label was incorporated into ATP and ADP. When the cells were incubated either with adriamycin or daunomycin both drugs inhibited the uptake of adenine in a dose dependent fashion (IC50 for adriamycin 20 $\pm$ 5 μM, for daunomycin 10 $\pm$ 5 μM). At these concentrations both drugs had very little effect on enzyme release. Moreover, the drugs inhibit adenine uptake without any delay while the effects on enzyme release induced by the drugs occurred at higher concentrations and was not detected for 2 hr for daunomycin and about 4 hr for adriamycin. Preincubation of the myocytes with daunomycin for up to 4 hr did not alter the inhibition of adenine uptake induced by the drug. Moreover, daunomycin decreased adenine uptake into the cells, but did not have any effect on the proportion of label incorporated into ATP-ADP or 5'-AMP. When the cells were incubated with daunomycin (55 μM, 4 hr) the capacity of the cell extracts to convert [^{14}C] adenine and PRPP to [^{14}C] 5'-AMP was not altered. This indicates that daunomycin at these concentrations does not have any effect on on the conversion of adenine into nucleotides but has a major effect on the uptake mechanisms.

In order to investigate whether daunomycin has similar effects on glucose as well as amino acid uptake, the cells were incubated with [^{14}C] deoxyglucose and [^{35}S] methionine. Daunomycin inhibited both deoxyglucose as well as amino acid uptake. However, there was a delay in the inhibition of deoxyglucose uptake while there was no delay in the inhibition of amino acid uptake.

Significance to Biomedical Research and Program of the Institute: The finding that daunomycin inhibits the uptake of adenine, glucose, as well as amino acids into cardiac myocytes indicates that the uptake of nutrients into the cells may be one of the early toxic manifestations of daunomycin as well as the other anthracycline anti-tumor drugs. Since the effect on uptake occurs at concentrations of drugs at which there are no gross membrane changes as evidenced by lack of enzyme release, the early inhibitory effects on uptake of nutrients may lead to the chronic toxicity observed *in vivo* with these drugs.

Proposed Course of Project: The inhibitory effect on the uptake of adenine, glucose and amino acids by cardiac myocytes will be investigated with other analogs of daunomycin and adriamycin in order to correlate these effects with the cytotoxic effects induced by these analogs.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00980-01 LCP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Induction of cytochromes P-450 by steroids

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Henry A. Sasame	Chemist	LCP	NHLBI
Other:			
Kiyoshi Nagata	Vist. Fellow	LCP	NHLBI
James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Dexamethasone and spironolactone in rats induce the same cytochrome P-450, (P-450p) as does pregnenolone 16 α-carbonitrile. Cytochrome P-450p possesses a high ethylmorphine N-demethylase activity in intact microsomes. This N-demethylase activity was blocked by antibody raised against cytochrome b5 comparable to the degree of inhibition of cytochrome c reduction mediated by cytochrome b5 and NADH cytochrome b5 reductase. Likewise these microsomes displayed regioselective induction of hydroxylation at 6 β-position of both testosterone and progesterone at the expense of hydroxylation of 16 α-position, suggesting that there is a specific cytochrome P-450 responsible for the hydroxylation. By analogy with the N-demethylase, the antibody against cytochrome b5 blocked preferentially the hydroxylation at the 6 β position of the steroids. In addition it also blocked the formation of an unidentified metabolite of testosterone.

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Project Description:

Objectives: Recently, Guzalian et al at Virginia Medical College have shown that dexamethasone and spironolactone induce a unique cytochrome P-450p which they have named cytochrome P-450p. They have isolated cytochrome P-450p liver microsomes of rats treated with pregnenolon 16 alpha carbonitrile or dexamethasone, but have not determined its substrate specificity. The N-demethylation of ethylmorphine by isolated cytochrome P-450p was extremely low and was inhibited by antibody raised against P-450p by only 20%. In view of the therapeutic usage of spironolactone and dexamethasone, it is of great value to study the nature of induction by these steroids.

Methods Employed: Conventional biochemical techniques have been applied. The separation of testosterone and progesterone metabolites was performed on Waters Associate HPLC equipped with a column in Radical Compression Separation System (Z-module).

Major Findings: 1) The rate of N-demethylation of ethylmorphine by liver microsomes from rats treated with spironolactone (SP), dexamethasone (DEX), or pregnenolone (16 α-carbonitrile (PCN) was 150% higher than that in microsomes from phenobarbital induced rats. Conversely the rate of N-demethylation of benzphetamine in liver microsomes from PB treated rats was higher than that in liver microsomes of rats treated with steroids. When liver microsomes from rats treated with PCN or dexamethasone were solubilized with cholate the loss of ethylmorphine N-demethylase activity was greater than that of benzphetamine.

2) Antibody raised against cytochrome b_5 inhibited the N-demethylation of ethylmorphine, codeine, aminopyrine and benzphetamine by liver microsomes or rats treated with PB or SP; the degree of inhibition paralleled the inhibition of cytochrome c reduction mediated by NADH cytochrome b_5 reductase.

3) Comparative studies of the metabolic profile of testosterone and PCN by hydroxylase microsomes revealed that treatment of the rats with PCN, DEX or SP results in regioselective differences in the hydroxylation of steroids. All the steroids increased the 6 beta hydroxylase with concomitant decrease in 16 alpha hydroxylase as measured with testosterone and PCN. The ratios 6 beta/16 alpha hydroxylase was 6-8, whereas the 6 beta/16 alpha hydroxylase ratios in liver microsomes from untreated and PB treated rats were 2.0 and 3.2, respectively. Moreover, the steroid treatment increased the formation of an unidentified metabolite of testosterone having an absorption maximum at 280 nm instead of 245 nm.

4) As with the N-demethyalse activity, the antibody against cytochrome b_5 blocked the hydroxylation at the 6 beta position and the formation of unidentified metabolite of testosterone by liver microsomes from untreated rats as well as those treated with steroids. The degree of inhibition was also comparable to the degree of NADH cytochrome b_5 mediated cytochrome c reduction.

Significnce to Biomedical Research to Program of the Institute: Both SP and dexamethasone are used as therapeutic agents for the treatment of a certain diseases. Since both drugs induce the same cytochrome P-450 as is induced by PCN in rats, it is worthwhile to investigate how these drugs affect the metabolism of endogenous substances such as steroids, fatty acids and protaglandins.

Proposed Course of Project: In order to pinpoint the exact nature of "induced cytochrome P-450" by these drugs, it is important to purify and characterize "induced cytochrome P-450" which possess a high specificity toward an endogenous substrates. Through the use of antibodies raised against these specific cytochrome P-450s, we hope to unravel the complexity of multiple effects manifested by different inducers.

Publications: None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00981-01 LCP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation and interaction of cytochromes P-450 in liver microsomes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Kiyoshi Nagata	Visiting Fellow	LCP	NHLBI
Other Investigator:			
James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Cytochromes P-450 plays a vital role in the oxidation of numerous foreign compounds (i.e. drug, carcinogens, insecticide and environmental polutants), as well as diverse endogenous substrates (i.e. Vitamin D, fatty acid, prostaglandin and steroids). Several cytochrome P-450 isozymes have been purified for various sources in the past few years. We have purified eleven cytochromes P-450 from hepatic microsomes from untreated rats and from rats treated with phenobarbital or 3-methylcholanthrene. Three or four of these eleven isozymes appear to be forms that have not been isolated previously. Based on immunochemical properties, these isozymes could be classified in five groups. We also found that eight of the eleven isozymes showed the high catalytic activities for foreign compounds and four of eleven isozymes showed the high catalytic activities for endogenous substrates.

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Project Description:

Objectives: Mammalian cytochrome P-450 systems in endoplasmic reticulum bound of liver comprise a flavoprotein (NADPH cytochromes P-450 reductase), cytochrome b_5, and a family of hemoprotein isozymes (cytochromes P-450) which catalyzes the oxidative metabolism of structurally diverse carcinogens, pollutants, insecticides and drugs as well as endogenous steroids, fatty acids and prostaglandins. Recently several cytochromes P-450 have been purified from liver microsomes. In order to study the regulation and interaction of thse isozymes of cytochrome P-450, we have purified several microsomal cytochromes P-450 from rat liver.

Methods Employed: The isozymes of cytochrome P-450 were purified as follows: Rat hepatic microsomes which were treated with 3-methylcholanthrene and phenobarbital and untreated were solubilized with sodium cholate. After centrifugation at 105,000 x g for 1 hr, the supernatant was applied to an ω-aminoacetyl Sepharase B affinity column. The isozymes were then eluted with 100 mM K-PO_4 buffer containing Emulgen 913. Further purification was achieved by passing the isozyme through an ion exchange column and a hydroxylapatite column.

The purified isozymes of cytochrome P-450 were characterized by determining their spectral properties, electrophoretic properties, immunochemical properties, peptide mapping and catalytic activities toward several substrates.

Major Findings: 1) Six cytochromes P-450 were purified from hepatic microsomes of rats treated with 3-methylcholanthrene treated rat hepatic microsomes. Nine cytochromes P-450 were purified from rats treated with phenobarbital and five cytochromes P-450 were purified from untreated. At least, eleven of these purified isozymes are distinct hemoprotein. 2) Three or four of eleven cytochromesP-450 appear to be isozymes that have not been isolated previously. 3) Antibodies against several of the isozymes have been prepared. Based on immunochemical properties, these isozymes were classified as five groups. 4) Eight isozymes showed the high catalytic activities for foreign compounds and four isozymes showed the high catalytic activities for endogenous substrates.

Significance to Biomedical Resarch and Program of the Institute: Cytochromes P-450 catalyze the oxidation not only of foreign compounds but also endogenous substrates. The cytochromes P-450 show the broad substrate specificity; some isozymes metabolize both foreign compounds and exogenous substrates. It is very important for biological functions to study the interaction and regulation of these isozymes

Proposed Course of Project: We are going to continue to purify cytochromes P-450 that have the high catalytic activity for endogneous substrate (fatty acids, prostagrandins and steroids). Our purpose is to clarify the interaction among cytochrome P-450 isozymes, cytochrome b_5 and NADPH-cytochrome P-450 reductase in microsomes by using these purified proteins.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 00982-01 LCP

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Purification of rat hepatic mitochondrial cytochrome P-450

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Ruth Chen	Staff Fellow	LCP	NHLBI
Other Investigators:			
James R. Gillette	Chief	LCP	NHLBI
Kiyoshi Nagata	Visiting Fellow	LCP	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH: Laboratory of Chemical Pharmacology

SECTION: Enzyme Drug Interaction

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Solubilized PB-induced rat hepatic mitoplasts (mitochondria without outer membrane hence with minimal microsomal contamination) containing mitochondrial cytochrome P-450 was chromatographed by successively on omega-aminoacetyl-sepharose 4B, DEAE sephocel, CM sepharose, and hydroxyapatite. Each chromatography was eluted with phosphate buffers of appropriate pH. Cytochrome P-450 content was determined by measuring the CO-difference spectra. The molecular weights of the cytochromes P-450 that were eluted from each column was monitored by SDS-PAGE. Four forms of mitochondrial cytochrome P-450 were isolated. The CO-difference maxima were 448-5, 449.0 449.5 and 450.0 nm.

Project Description:

Objectives: In the presence of NADPH both rat hepatic mitochondria and microsomes convert the hepatotoxin 1,1-dichloroethylene (vinylidene chloride, VDC) to monochloro acetate. The rate of formation of monochloroacetate (MCA) was greater in mitochondria than in microsomes. Since production of MCA by rat hepatic mitochondria and microsomes is catalyzed by isozymes of cytochrome P-450, these findings suggested that isozymes of mitochondrial cytochrome P-450 have a higher potential in metabolizing some xenbiotics than do those of microsomes. Our objective was to 1) purify the isozymes of mitochondrial cytochrome P-450 and 2) identify substrates that are preferentially metabolized by mitochondrial cytochrome P-450.

Methods Employed: Isolation of mitoplasts: Male Sprague Dawley rats (200 g) were treated with phenobarbital at 100 mg/kg for 4 days and then killed by decapitation. Livers were removed and homogenized with ice-cold 0.25 M sucrose (pH 7.4). The homogenates were then centrifuged at 600 g for 20 min. The supernatant was centrifuged at 8,800 g for 20 min. The pellet was carefully resuspended by a wide bore pasteur pipette in 0.25 M sucrose and centrifuged at 7,200 g for 20 min. The pellet was again carefully resuspended and centrifuged at 7,200 g for 20 min. The pellet was resuspended in 0.25 M sucrose at the concentration of 100 mg of mitochondrial protein per ml of sucrose. An equal volume of a solution of crystallized digitonin (1.25% in 2 mM Tris-HCl, 0.34 M sucrose, pH = 7.4) was added at 4°C to strip the mitochondria of its outer membrane. The mixture was stirred slowly for 10 min on ice and diluted with 3 volumes of Tris-sucrose (2 mM Tris-HCl and 0.34 sucrose, pH = 7.4) and gently homogenized. The homogenate was centrifuged at 8,800 g for 20 min. The mitoplast pellet was immediately solubilized with 30% glycerol, sodium cholate (1/3 of protein by weight) in 100 mM potassium phosphate (pH = 7.25).

Purification of mitochondrial cytochrome P-450. Cytochrome P-450 in solubilized PB-induced mitoplasts were purified by column chromatography at 4°C with additions of buffer A (20% glycerol, 0.1 mM DTT, 1mM EDTA, and 0.4% sodium chelate): 1) Omega-aminoactyl sepharose 4B. 100 mM potassium phosphate KPi; (pH = 7.25) in buffer A, 10 mM KPi (pH = 7.25) in buffer A, 10 mM KPi (pH 7.25) in buffer B, (20% glycerol, 0.1, MMDTT, 1 m MEDTA and 0.9% Emulgen) and 100 mM KPi (pH 7.25) in buffer C (20% glycerol, 0.1 mM BTT, 1 m MEDTA, 25% sodium cholate and 0.4% Emulgen were added in sequence. Four major bands of P-450 were separated. Content of P-450 was determined by measuring the CO-complex at 450 nm. Molecular weights were determined by SDS-PAGE. Cytochrome P-450 from each band was ultrafiltered through sephacel.

2) DEAE sephacel. Cytochrome P-450 from each band separated by omega-aminoacetyl sepharose 4B were further purified by elution and with 5,10,20,40,80 and 150 mM KPi (pH = 7.7) in buffer C. P-450 content and the molecular weight of each band were determined. P-450's of similar molecular weight were pooled, ultrafiltrated, and dialyzed.

3) CM sepharose. Buffer A with 5 mM KPi (pH 7.25) was added to this column to elute the dialyzed fractions from the DEA, sephacel separated P-450. Treatment of each eluted band followed above procedure.

4) Hydroxyapatite. Portions of buffer C with 5,10,20,40,80,300 mM KPi (pH = 7.25) were added to this column. The P-450 in each fraction was determined and subjected to electrophoresis, ultrafiltration, and dialysis.

Major Findings: Rat hepatic mitochondrial cytochrome P-450 with CO-difference spectra at peak heights of 448.5, 449.0,449.5, and 450.0 were purified. The specific amounts of the various cytochromes P-450, however, were very low.

Significance to Biomedical Research and Program of the Institute. It is known that most chemically reactive metabolites are formed by enzymes that we collectively called cytochrome P-450. In the past, this Laboratory has discovered the identify of several toxic metabolites formed by microsomal enzymes in liver. Purification of isozymes of mitochondrial cytchrome P-450 and identification fo substrate specificity of these isozymes broadens our understanding of the formation of reactive metabolites and the mechanism of their toxic action.

Proposed Course of Project: Loss of heme was found in the chromatographic purification of mitochondrial cytochrome P-450. An improved process of preventing heme loss should be developed. Appropriate substrate similar to the structure of VDC should be identified.

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTIONS ON CHEMICAL STRUCTURE AND STRUCTURAL
NUCLEAR MAGNETIC RESONANCE
NATIONAL HEART, LUNG AND BLOOD INSTITUTE

October 1, 1984 through September 30, 1985

This year the laboratory suffered a serious loss when Dr. John Pisano died on March 26, 1985. At the time of his death the section on Physiological Chemistry consisted of 5 full or part-time chemists working on the isolation, identification and function of physiologically important peptides. One professional, Dr. Jack Pierce has since retired leaving only one other full time doctoral level investigator, Dr. Hiroshi Nonoguchi who is currently working on peptide mediators in the regulation of renal tubular cyclic nucleotide metabolism and renal tubular water and electrolyte transport. These efforts initially involved bradykinin but more recently attention has switched to localizing the effects of atrial naturetic peptide (ANP) under the direction of Dr. V. Mangienello (LCN, NHLBI) and Dr. Mark Knepper (LKE, NHLBI).

Dr. Pisano's studies on isolation of new physiologically active peptides, notably the pipinins from Rana pipiens, have been completed and extended by his collaborator Dr. J. Osborne (LMD, NHLBI). For latest results in these areas, the annual reports of the above collaborators should be consulted. Dr. Pisano's kallikreinkinin studies have been summarized in a recent paper available from his collaborator Dr. Eric S. Marks (USUHS).

Further studies on peptides from Oxyuranus scutellatus, Heloderma suspectum and Megabombus pennsylvanicus will be conducted in Japan by his collaborator, Dr. H. Yoshida. Technical staff members of Dr. Pisano's section, P. Highet and A. Murphy will be reassigned shortly and M. DeLacy intends to leave government service.

The laboratory now consists of two groups, the Section on Nuclear Magnetic Resonance under Dr. Robert Highet and the Section on Chemical Structure under Dr. Henry Fales. Both are concerned with isolation, elucidating the structures and studying the properties of biologically important compounds.

Synthesis is a revived interest in the laboratory because we encounter so many requests for assistance in this area. Currently this activity is under Dr. Steve Miller, who has synthesized by a novel 2-step procedure an affinity ligand for human alcohol dehydrogenase. [4-(3-aminopropyl)-pyrazole] that is currently being used to separate isozymes of this system (P.R. Giri NIAAA). He has also synthesized 1-acetoxy -4-(2-trimethylammoniumacetyl)- naphthalene chloride from 1-naphthol in a search for a pH sensitive probe activated by intracellular esterases (I. Kurtz LKEM, NHLBI). While the fluorescence of this compound was to weak to be immediately useful, the synthetic approach is interesting.

Because synthetic organic chemistry this activity can command high salaries outside (38 K is average, 45 K from top schools, source: Chemistry & Engineering News, July 1985), it is anticipated that it will be necessary to use temporary staff fellow slots for this effort in the foreseable future.

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The Laboratory this year acquired 3 (!) nmr spectrometers. One, a 200 Mhz Varian XL-200, replaces the 60 Mhz JEOL which suffered from not being adaptable to modern pulse techniques. A second 300 Mhz Varian XL-300 instrument supplements our existing 300 Mhz spectrometer which is currently in constant demand with a considerable backlog of experiments. Finally, a third instrument, a 4.7 Tesla 33 cm bore General Electric animal spectroscopy and imaging spectrometer to be located in Building 1, represents an experimental design and will allow both basic spectroscopic studies and imaging (R. Balaban LCEM, NHLBI). The first instrument has arrived and is operating satisfactorily, the second is being installed now while the third awaits renovation of Building 1 in the fall of 1985.

Initial experiments done by J. Ferretti and R. Balaban on the General Electric spectrometer in California, using an anesthetized rabbit leg, yielded good spectra showing sufficient resolution and sensitivity. Imaging of a rabbit eye using a surface coil showed clearly resolved blood vessels.

In NMR this year, J. Ferretti and R. Balaban (LKEM, NHLBI) report an interesting study of adenosine triphosphate with adenylate kinase showing by 2-D-^{31}P-NMR, formation of the unexpected linear adenosine tetraphosphate identical to that found in equine muscle. Formation of this compound is catalyzed by adenosine diphosphate, a routine impurity in ATP preparations.

Basic to all NMR studies is the determination of errors in relaxation time measurements. Ferretti has completed (G.H. Weiss, PSL, DCRT) a thorough study of all sources of error and found one remaining even in the absence of instrumental noise. This is under investigation. Similarly, a perturbation expansion solution to the Bloch equation has been developed and examined under conditions of strong RF fields to demonstrate the region in which the solutions are valid.

In other studies Ferretti has completed a detailed nuclear Overhauser effect (NOE) study to determine in solution the distances between atoms in an actinomycin related peptide, (T. Mauger, Washington Hospital Center), using a new 2-D technique in the absorption mode. The results refute an earlier conformation model based on rates of deuterium exchange.

R. Highet has shown, using NMR, that phloroglucinol forms a tris bisulfite addition compound in solution. Attempts at isolation failed and characterization depended entirely on ^{13}C and ^{1}H NMR. He has also studied a series of toxic mold metabolites from Alternaria alternons (M. Stock, E. Mazzola, S. Page, FDA). These complex aromatic epoxyquinones undergo a series of peculiar reactions with reducing agents and solution of their structures relied heavily on modern pulse NMR data. Two more bile metabolites of bromobenzene (5-and 6-glutathionyl-2-bromohydroquinone) have also been solved structurally using NMR (S. Lau, T. Monk, Georgetown Univ).

In mass spectrometry, the ^{252}Cf plasma desorption spectrometer continues to provide excellent spectra and is in continuous use due largely to the efforts of E. Sokolosky and L. Pannell, the latter now with NIADDK. A computer operated electromechanical mechanical device allowing remote (from home) sample-changing greatly facilitates running the maximum seven samples overnight and a newly designed sample holder reduces likelihood of sparking to the dangerous radioactive foil. This safety device has been supplemented with a system providing

positive shutdown of high voltage and isolation in case of pump or electrical failure.

In ^{252}Cf PDMS spectra, experience with a wide variety of sample types is beginning to provide a picture of the processes involved and prediction of success with unknowns (in terms of quantity and type) is improving. Series of compounds providing useful spectra and involved in solving pertinent questions include: functionalized xanthine and adenosine derivatives used in pharmacological experiments (K. Jacobson, NIADDK), an antimalarial peroxide and derivatives (D. Klayman, USWRAMC, A. Brossi, NIADDK), cord factor (trehalose mycolates) (M. Goren, Jewish Hospital, Boulder, CO), an endogenous diazepine receptor (A. DeBlas, Stoneybrook, NY), aromatic glycosides (V. Feil, USDA, Fargo, ND), halogenated uracils and uridines, digitonin components, cortex and papillary fluids, (R. Balaban, NHLBI), propylene oxide adducts of cyclodextran and digitonin (J. Pitha, NIA), aromatic and polyolefinic compounds, and a series of 10-15 amino acid peptides (J. Rivier, U of C).

In the natural products area, H. Lloyd has completed structural analysis of a compound isolated in the Amazon in 1975. This substance, a salt of pyrrolle -3-carboxamidine was isolated with great difficulty by HPLC and characterized by nmr and mass spectrometry of the free base and its diacetate. Synthesis by a known but difficult procedure supplied enough compound to further establish the structure and provide assurance that the main physicological activity (convulsant) was indeed due to this compound. We regard this as a very unusual structure both from a pharmacological and botanical standpoint.

Other analogs such as the 2-isomer, 3-indolyl-, 2,3,4-pyridyl-derivatives have been prepared and none have the described physiological activity.

In related natural products studies, using HPLC and PCC we have separated and identified (using ^{252}Cf PDMS) seven components of commercial digitonin including one apparently unreported glycoside. In the pheromone area, Lloyd has found *cis* and *trans* Solenopsin A and its tridecyl analogs in *S. maniosa* while anabasine was found for the first time in an animal *Messon ebeninis*. *Reticulitermes* secretions were found to contain γ -cadinene, tetradecyl propionate and unsaturated hydrocarbons and *Cicindela flexuosa* uses a mixture of tetradecyl- and hexadecyl acetates in its defense.

In x-ray crystallography, J. Silverton has implemented the MITHRAL direct methods programs successfully on the NIH IBM central computer. The next logical extension is to place these routines on an IBM-PC-AT laboratory computer effecting considerable savings. This will be attempted shortly and may be the beginning step in setting up a network of Laboratory personal computers to achieve ultimate independence from DCRT, (since this is their aim also). Experience on laser printers by Silverton has been shared in DCRT's "Interface" and undoubtedly will be useful to the Institutes as they acquire these faster systems.

Silverton has completed the following extensive series of x-ray structural analyses this year: Thisemicarbazides and metal complexes (J. Scovill, WRAMC and A. Bavoso, U. of Naples), racemic-p-fluorophenylalanine (correllation with NMR results), (D. Torchia & Y Hiyama, NIDR), conformation of peptides related to actinomycin (correllation with NMR results), (T. Mauger, Washington Hospital

Center & J. Ferretti), calcium/EGTA (R. Berger, NHLBI), qinghaosu (artemesin), (A.J. Lin and D. Klayman, WRAMC), conformation of medium sized rings (H. Ziffer & S. Ito, NIADDK), a naphthalene-cyclophane clathrate of pyridine (S. Miller, NHLBI), colchinoids (A, Brossi & R. Dumont, NIADDK).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01003- 13 CH

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure of Natural Products Using Instrumental Methods

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory and institute affiliation)*

P.I.	H.M. Fales	Chief, Laboratory of Chemistry, NHLBI, CH
OTHER	Y.M. Yang	(Visiting Fellow, Shanghai, PRC)
	E. Sokoloski,	Laboratory of Chemistry, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cf-252 mass spectrometry has been applied to a wide variety of problems including synthetic intermediates, peptides, natural products isolated at NIH, drug metabolites, kidney fluids, glycosides and digitonides. Insect studies have been carried out on Camponotus vagans.

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1. Extensive experimentation has been conducted with the Cf-252 plasma desorption spectrometer. The spectrometer has now been modified so that samples may be changed automatically from a remote location using an RS-232 interface. This enables us to inspect and run several samples each night. A very complete safety system has been installed, providing instant shutdown of high voltage upon pump or electrical failure. Spectra have been run as follows:

a. A series of complex functionalized xanthine and adenosine derivatives have been studied. All provided good molecular ions; incomplete reactions and unusual by-products were detected (K. Jacobson, NIADDK).

b. The antimalerial peroxide quinhaosu (artemesinin) and its dihydro derivative have reavealed unusual spectra showing abundant $(2M+H)^+$ ions and loss of formate from the negative ion, D. Klayman (USAWRAMC), A. Brossi (NIADDK).

c. Many derivatives related to "cord factor" (trehalose mycolates) have provided molecular weights of homologous series allowing a very complete picture of a complex problem, (M. Goren, Jewish Hospital, Boulder, Colorado).

d. An endogenous diazopine receptor sample has been identified as N-demethyldiazapam using both Cf-252 PDMS and confirmed using E1 mass spectrometry (A. DeBlas, Stoneybrook, LI, N.Y.)

e. A series of aromatic glycosides has been examined. All provide excellent $(M+Na)^+$ ions. (V. Feil, USDA, Fargo, N.D.).

f. For comparision with FAB mass spectrometry, a series of halogenated uracils and uridines has been investigated. In common with the FAB method, all show loss of halogen decreasing in the order I>Br>Cl>F, but less overall loss is observed

g. A complete separation of commercial digitonin into its components has been carried out using the Ito planetary coil centrifuge. All components were characterized by abundant $(M+Na)^+$ ions in their Cf-252 PDMS spectra.

h. A study of raw cortex and papillary fluid extract was conducted using PDMS. Besides the expected NaCl & KCl, betaine was easy to detect. Other uncharged derivatives (sorbitol, inositol, glycerophosphoric acid, etc) were detected in the more classical manner using trimethylsilylation and GCMS (R. Balaban, NHLBI).

i. An extensive series of propylene oxide adducts of cyclodextran and digitonin was studied to determine the degree of substitution of the aliphatic hydroxyls. The results check other methods (nmr, combustion analyses) in most cases and the technique is now regarded as the method of choice. The cyclodextrans are used in solubilizing drug preparations (J. Pitha, NIA).

j. A series of aromatic and polyolefinic compounds have been studies to determine if $M^{+\cdot}$ or $(M+H)^+$ ions are formed. As in FAB spectrometry, the former are more pronounced when the ionization potential is low.

k. A series of peptides containing 10-15 amino acids has been run successfully to check sequences of material submitted for human testing (J. Rivier, U. of Cal).

2. Insect studies have been minimal this year. The cowpea weevil pheromone W. Burkholder, U. of Wisconsin) is still under study but no progress has been made because the very small quantities available (about 5-10 ug) are in a complex matrix of much larger quantities of fatty acids. Males of the carpenter bee Componotus vagans have been found to contain mellein, 6-methyl methylsalicylate farnesene and a phytadiene while the females and workers, only traces of mellein. The function of these pheromones is still under investigation.

PUBLICATIONS

1. Jones, T.H., Highet, R.J., Blum, M.S., and Fales, H.M.: (5Z,9Z)-3-Alkyl-5-methylindolizidines from Solenopsis (Diplorhoptrum) Species. J. Chem. Ecol. 10: 1233-1249, 1985.

2. Fales, H.M., Pannell, L.K., Sokoloski, E.A.: Separation of Methyl Violet 2B by High-Speed Countercurrent Chromatography and Identification by Californium-252 Plasma Desorption Mass Spectrometry. Analytical Chemistry 57: 376, 1985.

3. Kelner, L., Markey, S.P., Fales, H.M., Lundquist, T.R.: Secondary Emission Mass Spectrometer, Application and Evaluation: International J. of Mass Spectrometry and Ion Processes. 62: 237-251, 1984

4. Lloyd, H.A., Fales, H.M., Goldman, M.E., Jerina, D.M., Plowman, T., Schultes, R.E.: Brunsfelsamidine: A Novel Convulsulant From the Medicinal Plant Brunfelsia Grandiflora. Tetrahedron Letters. 26: 2623-2624, 1985.

5. Pannell, L.K., Fales, H.M., Scovill, J.P., Klayman, D.L., West, D.X., Tate, R.L.: Plasma Desorption Mass Spectrometry of Transition Metal Complexes of Thio- and Selenosemicarbazones of 2-Acetylpyridines: Transition Met. Chem. 10: 141-147, 1985.

6. Yi-Ming, Y., Fales, H.M., Pannell, L.K.: Dehalogenation Reactions in Californium-252 Plasma Desorption Mass Spectrometry. Anal. Chemistry, 57: 1771-1772, 1985.

7. Fales, H.M., McNeal, C.J., Macfarlane, R.D.: Californium-252 Plasma-Desorption Mass Spectrometry of Polymethylenediamine-Linked Enkephalin Peptides. Anal. Chemistry. 57: 1616-1621, 1985.

8. Pannell, L.K., Sokoloski, E.A., Fales, H.M., Tate, R.L.: Californium-252 Plasma Desorption Mass Spectrometry of Cationic, Anionic, and Neutral Dyes. Anal. Chemistry. 57: 1060-1067, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01004-14 CH

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Natural Products

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Helen A. Lloyd	Research Chemist	NHLBI, CH

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The work involves the structure determination of physiologically active compounds of plant and animal origin. Various types of chromatography (gas, thin layer, ion exchange, liquid) are used to isolate pure samples of unknowns. Structures are determined by chemical methods (degradation and synthesis) and with the aid of spectrometry (infra-red, UV, NMR and mass spectrometry).

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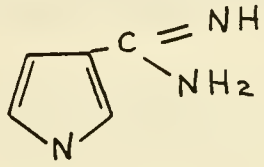
1. Physiologically active plant products.

a) The screening of potential carcinogenic plants (ex. Krameria, Acacia, Sesamun and Acacia species) was continued (with Govind Kapadia, Ph.D., Howard University). Mass spectra and the interpretation of the data were provided.

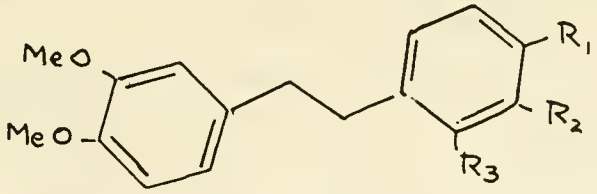
b) Structure of brunfelsamidine, a convulsant from Brunfelsia grandiflora (with H.M. Fales and M.E. Goldman, CH/NHLBI). Extracts of this plant are widely used by natives of the Amazon region as medicine, narcotic and fish poison. Ingestion of the water extracts induces analgesic and hallucinogenic effects with sensations of chills. A water extract of Brunfelsia was chromatographed on a CM-Sephadex column and the fractions were monitored biologically (intraperitoneal injection in mice). A fraction inducing convulsions was further purified and obtained as a crystalline hydrochloride. Examination of the NMR and MS of the compound and its diacetate showed that it was either pyrrole-2-carboxamidine or the 3-carboxamidine isomer. Both amidines were synthesized through the corresponding nitriles. The 3-isomer (1) was identical to the natural product.



This is a very novel compound in view of the rarity of 3-substituted pyrrole derivatives in nature. Studies on the pharmacology of this substance are in progress but its mode of action is still unclear. Analogs such as indole, pyridine and furane derivatives have been synthesized for biological testing.

2. Synthetic drugs and analogs.

a) A project involving synthetic dopaminergic and hypotensive drugs related to (2) was continued (with Stanley Evans, Ph.D., Meharry Medical College) with the mass spectrometric studies conducted in this laboratory.



b) HPLC separations and mass spectra interpretation of benzophenanthridine, 3-arylisochromene and 3-arylisoquinoline analogs of naturally occuring alkaloids were accomplished. (I.W. Elliot, Ph.D., Fisk University).

c) Studies on digitonin (with H.M. Fales and Y.M. Yang, CH/NHLBI) Samples of commercial digitonin (from Fluka and Sigma) were examined by HPLC and shown to be mixtures of six or seven components. The impurities were separated by preparative HPLC and their structures determined by Cf-252 plasma desorption mass spectrometry, hydrolysis

and identification of the aglycone and sugar moieties. Previous mass spectrometric studies of digitonin by other authors were based on the assumption that the commercial samples were homogenous.

3. Insect Pheromones.

Determination of structure of unknowns using mostly GC-MS. (with Abraham Hefetz, Tel-Aviv University: Murray Blum, University Jean-Luc Clement, Universite Pierre et Marie Curie, Paris, France).

a) The poison gland products of the ant Solenopsis maniosa were identified as the alkaloids cis and trans-2-undecyl-6-methylpiperidine in a 1.2:1 ratio. Traces of the cis and trans isomers of the 2-tridecyl compound occur as concomitants.

b) The alkaloid anabasine (2-(3-pyridyl) piperidine) was identified as the main product of the poison glands of the ant Messor ebeninus. This compound had not been observed previously in animals.

c) Various species of Reticulitermes termites were investigated. Their secretions were shown to contain γ-cadinene and a corresponding alcohol (R. flaviceps), geranyl linalool (species RF2), tetradecyl propanoate (R. lucifugus) and various unsaturated branched hydrocarbons.

d) The defensive secretion of the tiger beetle Cicindela flexuosa was characterized as a mixture of tetradecyl acetate and hexadecyl acetate.

PUBLICATIONS

1. Hefetz, A., Lloyd, H.A., Valdenburg, A. The defensive secretion of the tiger beetle Cicindela flexuosa (Cicindelinae; Carabidae). Experientia. 41: 539, 1984

2. Lloyd, H.A., Fales, H.M., Goldman, M.E., Jerina, D.M., Plowman, T., Schultes, R.E.: Brunfelsamidine: A novel convulsant from the medicinal plant Brunfelsia grandiflora. Tet. Letters. 26: 2923, 1985.

3. Blum, M.S., Jones, T.H., Lloyd, H.A., Fales, H.M., Snelling, R.R., Lubin, Y., Torres, J.: Poison gland products of Solenopsis and Monomorium species. Jour. Ent. Science. In press.

4. Lloyd, H.A., Schmuff, N.R., Hefetz, A.: Chemistry of the anal glands of Bothriomyrmex syrius Forel. Olfactory mimetism and temporary social parasitism. Comp. Biochem. and Physiol. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01005-14 CH

PERIOD COVERED

October 1, 1984 to September 31, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Solid State Studies of Physiologically Important Molecules.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: J. V. Silverton Research Chemist CH NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER.
1.1	1.1	0.00

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The solid-state work of the Laboratory of Chemistry, NHLBI concerns structural investigations and computer calculations on biologically interesting compounds. Drug action, biological uses of metal compounds and conformational studies on peptides and medium ring compounds have been carried out.

397

Project Description

a) Thiosemicarbazides and their metal complexes (with Dr. J. Scovill, WRAMC and Dr. A. Bavoso, University of Naples).

In the course of writing up this work for publication, it was necessary to reassess the interesting observation of an unexpected solid state tautomer. The explanation appears to be related to its suitability for hydrogen bond formation. Quantum mechanical (CNDO) calculations appear to support the explanation and a manuscript is expected to be submitted for publication within a few weeks.

b) Racemic p-fluorophenylalanine (with Drs D. Torchia and Y. Hiyama, NIDR).

The completion of the solid state NMR work has allowed correllation of solid state results by x-ray and NMR and the unusual spectroscopic results and physical properties including twinning, are consistent with both modes of investigation. A manuscript has been submitted to J. Am. Chem. Soc.

c) Peptide conformations: peptide lactones related to actinomycin (with Dr. T.A. Mauger, Washington Hospital Center and Dr. J.A. Ferretti, NHLBI-LC).

The correllation of solid state and solution studies, both static and kinetic, has been completed and a paper has been accepted for publication in J. Am. Chem. Soc. It is impossible to resist quoting one review, "an exceedingly fine paper that deserves immediate publication in JACS. I recommend this paper be published without delay".

d) Calcium EGTA compexes (with Dr. R. Berger, Laboratory of Technical Development NHLBI).

EGTA, because of its specificity for calcium over magnesium, is the calcium buffer of choice in biological investigation. However the nature of the calcium complex is unknown as are the reasons for specificity. Attempts have been made for several years to prepare crystal suitable for x-ray investigation but, despite much preparative and preliminary crystallographic work, the results have been most frustrating. Some observations have been interesting but not perhaps to the point; the unusual mineral Gaylussite, Calcium sodium carbonate, was made and identified by x-ray methods. Its crystal structure is known and the work was not repeated. Some details are emerging as to the variation of chemical composition with pH; under acidic conditions crystals were formed which proved to be EGTA itself without complexed calcium. Since the structure was unknown, x-ray data were collected and the structure was solved (in truth, the previously un-reported crystals were identified by carrying out a crystal structure determination because of unavoidable delays in analysis). The structure proves to be zwitterionic and the crystals possess interesting hydrogen bonding whose details are being studied. Attempts are being made to prepare the calcium complex by newer and perhaps simpler techniques than previously employed.

e) Conformation of medium-sized rings (with Drs. H. Ziffer and S. Ito, NIADDK).

The crystal structures of four compounds of this type have been investigated to provide a basis for molecular mechanics calculations. An absolute conformation

determination was also carried out to provide fundamental results for the series. The molecular mechanics are still in progress and are being carried out with the aid of a computer compatible with the IBM PC. In addition to the basic investigation, useful experience is being gained in the use of small computers for such work.

f) Qinghaosu (with Drs. A.J. Lin, D. Klayman and J.R. Hoch, WRAMC).

Artemisin (quinghaosu) is a natural antimalarial long known in Chinese traditional medicine. A determination of the solid state structure and absolute configuration of a derivative by the anomalous scattering of oxygen has been completed. The work has been submitted for publication.

g) Siderophoric metal complexes (with Drs. O. Gansow and M. Magerstadt, NCI).

Considerable preliminary work has been carried out but, so far, only one well crystallized complex has been obtained; a bismuth compound. The x-ray data will be collected soon. It may be necessary to use short-wave length molybdenum radiation but the possibility of a measured absorption coefficient is being considered (perhaps the data may be collected both ways since no real comparison of the methods seems to have been done).

h) Further work on colchinoids (with Drs. A. Brossi and R. Dumont, NIADDK).

An absolute configuration by x-ray methods has never been carried out for colchicine and, when a suitable crystal is prepared, we propose to do it. The chemical determination is probably reliable but it was surprising, in view of the amount of work in the crystallographic literature, to find no determination there of absolute configuration (the depicted configuration is usually that determined chemically but the coordinates given do not always correspond to it).

The racemic form of colchicine has a high melting point and it seemed interesting to investigate. After a fair amount of work, the very soluble compound was crystallized. The crystals proved to include two molecules of water. There is an interesting pattern of hydrogen bonds and this and the molecular conformations are being assessed.

i) A naphthalene-cyclophane clathrate complex of pyridine (with Dr. S. Miller of this laboratory).

A structure investigation of the rather large molecule and complicated crystal has been completed and the results are being considered.

j) Acid rearrangment products of narcotic agonists (with Dr. E.L. May, Medical College of VA and Dr. K.C. Rice, NIADDK).

This is a project involving structure confirmation and absolute configuration and is almost complete.

k) Computational aspects.

The latest package of direct methods programs (MITHRIL, Dr. C. Gilmore,

Glasgow University) has been implemented on the NIH IBM central computer. The implementation went smoothly and there appear to be few obvious bugs in the system although one or two minor ones were found. A further step in the decentralizing of computation will be to try to use MITHRIL on an IBM PC-AT. The small computer can be obtained with adequate memory for this purpose and one has been ordered. It is also hoped to set up a laboratory local network using such machines since there are many indications that such will be common in the near future.

Experience with the TEK 4112B terminal was enhanced by taking a course on local programmability and it may be possible to link this terminal to an IBM network, perhaps by means of a modem since the operating system of the 4112B is CP/M.

Procedures for use of the laser printers of the central computer for document preparation have been refined and experience has been shared with other users both by means of the DCRT "Information Exchange" and by an article in the DCRT publication: INTERFACE (no. 122, February 1985).

PUBLICATIONS.

1) Probes for narcotic receptor mediated phenomena 3. Oxide-bridged 5-phenylmorphans. Burke, T.R., Jacobson, A.E., Rice, K.C., Weissman, B.A., Silverton, J.V.: NIDA Research Monograph 49: 109-13, 1984.

2) Synthesis and binding to tubulin of colchicine spin probes. Sharma, P.A., Brossi, A., Silverton, J.V., Chignell, C.F.: J. Med. Chem. 27: 1729-33, 1984.

3) Probes for narcotic receptor mediated phenomena 6. Burke, T.R., Jacobson, A.E., Rice, K.C., Silverton, J.V.: J. Org. Chem. 49: 2508-10, 1984.

4) Sterochemistry of PN bond cleavage. First crystal and structural assignment in cyclic phosphoramidofluoridates. Misiura, K., Silverton, J.V., Stec, W.J.: J. Org. Chem. 50: 1815-1818, 1985.

5) Structure of (7S)-N-acetylcolchicine. Silverton, J.V., Sharma, P.A., Brossi, A.: Acta Crystallographica. 41C: 7455-758, 1985.

6. Structure of 1-demethylcolchicine. Sharma, P.A., Brossi, A., Silverton, J.V. Acta Crystallographica. 41C: in press, 1985.

7) Conformation and dimerisation of actinomycin related peptide lactones in solution and in the solid state. Mauger, A.B., Stuart, O.A., Ferretti, J.A., Silverton, J.V. J. Am. Chem. Soc. 107: in press, 1985.

400

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HL 01006-14 CH

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Characterization of Natural Materials and Metabolic Products

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Robert J. Highet Research Chemist CH NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

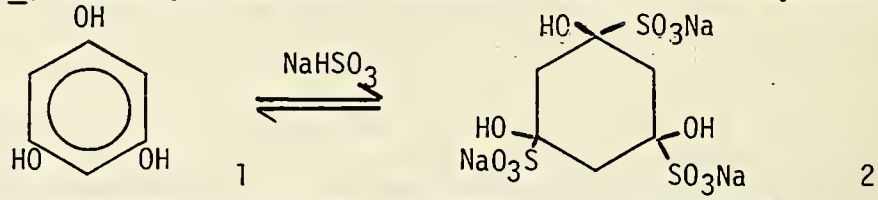
SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Structural studies have been completed on a series of metabolites from the mold Alternaria alternans and on bile metabolites of bromobenzene. Investigation of phloroglucinol tautomeism has been extended to the sodium bisulfite addition compound.

4 8 1

1. Studies on the tautomeric properties of phloroglucinol

Early reports have described the preparation of a bisulfite addition compound of phloroglucinol which is remarkably stable to acid. Reinvestigation has shown this material actually to be 3,5-dihydroxybenzene sulfonic acid. Phloroglucinol, 1, actually forms the trisbisulfite addition compounds,



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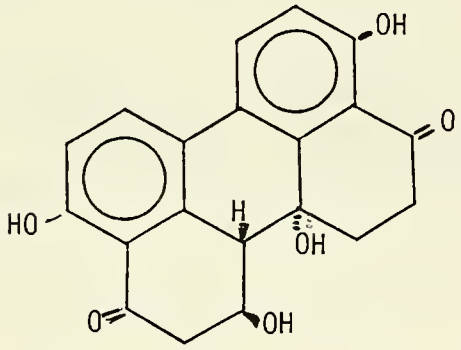
2, spontaneously, but with an equilibrium constant of approximately 2. Inasmuch as attempts to prepare conventional derivatives have failed, the characterization has depended on proton and carbon-13 nmr spectral studies.

A collaborative study with Mr. T. Jaouni of this laboratory has shown that 2,4,6-trimethylphloroglucinol resembles the parent compound 1 in forming an alicyclic dianion.

2. Structural studies on mycotoxins from Alternaria spp.

Collaborative investigations with Mr. Michael Stack and Drs. Eugene Mazzola and Samuel Page of the Food and Drug Administration have suggested structures for three toxic products from the common mold Alternaria alternans. Altertoxins I and II correspond to structures 3 and 4. However, reduction by



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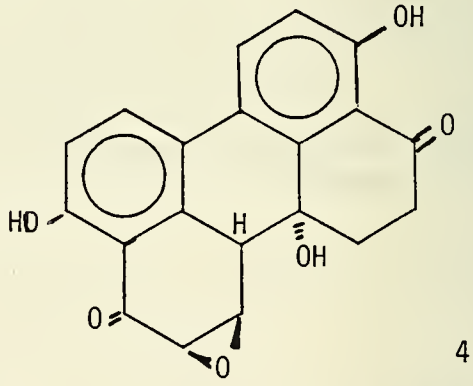

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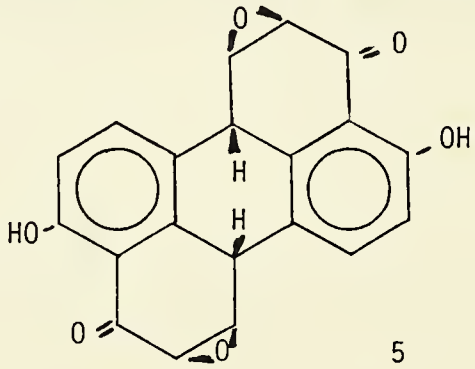
sodium borohydride of Altertoxins I and III provides products with substantially different ultraviolet spectra, that from III resembling the absorption from the phenol anion itself. Accordingly, 5 is suggested for the structure of Altertoxin III.



5

402

Evidence for the presence of epoxide group is found largely in the large one-bond proton-carbon coupling, of 185 Hz.

3. Bile Metabolites

In a continuing collaboration with Dr. Serrine Lau of the National Cancer Institute and Dr. Terrence Monk of Georgetown University, two more bile metabolites of bromobenzene have been identified as the 5- and 6-glutathionyl-2-bromohydroquinone.

Publications

1. Monks, T.J., Lau, S., Highet, R.J.: Formation of Nontoxic Reactive Metabolites of p-Bromophenol. Drug. Metab. and Disp. 12: 432-7, 1984.

2. Branchflower, R.V., Nunn, D.S., Highet, R.J., Smith, J.H., Hook, J.B., Pohl, J.R.: Nephrotoxicity of Chloroform: Metabolism to Phosgene by the Mouse Kidney. Toxicol. Appl. Pharmacol. 72: 159-168, 1984.

3. Daly, J.W., Highet, R.J., Myers, C.W.: Occurrence of So-called Denrobatid Alkaloids in Phyllogentically Unrelated Anurans of Brazil (Bufonidae), Malagasay (Mantellidae), and Australia (Leptodactylidae), Toxicon, 22: 905-919, 1984.

4. Highet, R.J., Wheeler, J.W.: The Study of Alkaloid Structures by Spectral Means, in "The Alkaloids". (A. Brossi, Ed). 24: 187-348, 1985.

5. Ferretti, J.A., Highet, R.J., Pohl, L.R., Monks, Y.J., Hinson, J.A.: Two-Dimensional J-Resolved Nuclear Magnetic Resonance Spectral Study of Two Bromobenzene Glutathion Conjugates, Environmental Health Perspectives, in press.

6. Monks, J.J., Lau, S.S., Highet, R.J., Gillette, J.R. Glutathione Conjugates of 2-Bromohydroquinone, A Metabolite of Bromobenzene, Are Nephrotoxic. Drug. Metab. and Disp, in press

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01027-03 CH

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Nuclear Magnetic Resonance Spectroscopy on Biologically Important Molecules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

James A. Ferretti Research Chemist NHLBI CH

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER.
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Development and application of multiple pulse Fourier transform methods in Nuclear Magnetic Resonance Spectroscopy. Applications include enzyme catalyzed rate studies in vitro and in vivo as well as structural and conformational properties of antibiotics, toxins, peptides and small proteins in solution.

PHS 6040 (Rev. 1/84) GPO 904-917

1. Enzymatic Reactions and In Vivo NMR

The application of NMR spectroscopy to in vivo studies is an ongoing project. Experimental protocols have so far been limited to 150g rats using a home built probe in an 89 mm bore magnet. The General Electric 33 cm bore 4.7 Tesla animal spectroscopy and imaging system is in its final design and testing stages and should be ready for shipment to NIH in October. Preliminary studies on the system carried out in California in collaboration with Dr. Robert S. Balaban (LKEM, NHLBI) indicate that the system is quite versatile and is meeting specifications. Resolution and sensivity studies on the leg of an anesthetized rabbit yielded good quality phosphorus spectra in times under one minute. Initial imaging studies have also been performed on the system. Preliminary surface coil images on the eye of an anesthetized rabbit showed clearly resolved blood vessels.

A study of binary complexes of adenosine triphosphate and adenylate kinase was initiated in collaboration with Dr. V.V. Kupriyanov of the Institute of Experimental Cardiology of the U.S.S.R. and Dr. Balaban. Assignments of the resonances of the binary complexes have tentatively been made. During the course of this investigation, the slow formation of an unidentified compound was observed. Characterization of the compound, which was carried out using two-dimensional homonuclear phosphorus-31 correlation spectroscopy, showed that it was linear adenosine tetraphosphate. This metabolite is identical to that found in equine muscle. The mechanism of formation was subsequently investigated and found to be the adenylate kinase catalyzed reaction of adenosine triphosphate with adenosine diphophate, which is always found as an impurity in preparations of adenosine triphosphate, to form the adenosine tetraphosphate and adenosine monophosphate.

2. Theory and Precision in Nuclear Magnetic Resonance Spectroscopy.

In collaboration with Dr. G.H. Weiss (PSL, DCRT) an experimental determination of the errors in spin-lattice relaxation time (T_1) measurements are being carried out. Analysis of the data has included all sources of error and also consideres effects of correlations in the noise due to filtering and Fourier transformation. The measurements also show that there is error in the estimation of T_1 even in the absence of instrumental noise. The sources of this error is being investigated.

A perturbation expansion solution to the Bloch equations for rapid scan correlation NMR spectroscopy has been developed. In this study the theoretical basis for the use of cross correlation to the extract an undistorted spectrum from the rapid scan response is examined under conditions where strong radio frequency fields are employed. The application of strong radio frequency fields results in a nonlinear response by the nuclear spin system which requires the perturbation expansion treatment. A comparison of the results with those obtained by a numerical solution of the Bloch equations demonstrated the region in which the peturbation solution was valid.

An investigation of the use of the maximum entropy (minimum information) method to extract one- and two-dimensional spectra from the related free induction delays has been initiated. Preliminary results suggest that no new information over that obtained using optimum filters is obtained.

3. Structural and Conformational Studies using Modern Pulse NMR Methods.

Additional studies of the conformation of actinomycin related peptide lactons are being carried out. These studies are being done in collaboration with Dr. A. B. Mauger (Washington Hospital Center). A detailed nuclear Overhauser effect study to determine the non bonded intramolecular pistan distances in has been completed. This study that a previously proposed conformation based on dexterium exchange data is incorrect. In the course of this study it was necessary to implement a new two dimensional technique which allowed spectra to be recorded in absorption (as opposed to absolute) mode.

The assignments of the NMR spectra of a set of toxins (altertoxins I, II, and III) are being carried out using a combination of new pulse techniques. This study is in collaboration with Dr. Robert J. Highet. Other studies on cell extracts are in progress.

PUBLICATIONS

1. Weiss, G.H., Ferretti, J.A.: The Choice of Optimal Parameters for Measurement of Spin-Lattice Relaxation Times. III Mathematical Preliminaries for Nonideal Pulses. J. Magn. Resonance. 61: 490-498, 1985.

2. Weiss, G.H., Ferretti, J.A.: The Choice of Optimal Parameters for Measurement of Spin-Lattice Relaxation Times. IV Effects of Nonideal Pulses. J. Magn. Resonance. 61: 499-515, 1985.

3. Ferretti, J.A., Weiss, A.K., Weiss, G.H.: Errors in the Measurement of NDE Factors. J. Magn. Resonance. 62: 319-321, 1985.

4. Bax, A., Ferretti, J.A., Nashed, N., Jerina, D.M.: Complete H and 13c NMR Assignment of Complex Polycyclic Aromatic Hydrocarbons. J. Org. Chem. in press 1985.

5. Mauger, A.B., Stuart, O.A., Ferretti, J.A., Silverton, J.V.: Conformation and Dimerization of Actinomycin Related Peptide Lactons in Solution and in the Solid State. J. Amer. Chem. Soc. in press 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01028-01 CH

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Synthesis and Characterization of Bioactive Materials

PRINCIPAL INVESTIGATOR *(List other professional personnal below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Stephen P. Miller Research Chemist CH NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.9	0.9	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Bioactive compounds were studied by a combination of chemical synthesis and instrumental analysis.

1. Synthesis:

a) An affinity ligand for human alcohol dehydrogenase (4-(3-aminopropyl)-pyrazole) was synthesized via a two-step procedure for use in enzyme purification.

b) A previously unreported "molecular probe" derived from 4-acetyl-1-naphthol was prepared. It's uptake and cellular fate were studied in tissue cultured cells by fluorescence microscopy.

2. Analytical Studies: a) Experiments are being conducted to determine the structure of the Cowpea weevil pheromone using a combination of microchemistry and GC/MS. This insect is an important pest of the blackeyed pea. b) Our plasma desorption mass spectrometer allows us to obtain molecular weight information on a series of complex (MW=1000-1800) and highly involatile sugar-containing saponins. These compounds are being studied by French collaborators as the growth inhibiting substances present in alfalfa (Medicago sativa). c) A single-crystal x-ray diffraction study was undertaken (with J. Silverton CH NHLBI) to determine the exact dimensions of a naphthalene cyclophane. This compound is a model for an unfunctionalized hydrophobic cavity.

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A. Synthesis of 4-(3-aminopropyl)-pyrazole April 1985 - July 1985 (in progress). In collaboration with P.R. Giri, Laboratory of Genetics, NIAAA, NIH.

The tille compound is a tight binding inhibitor of mammalian alcohol dehydrogenase enzymes, and possesses suitable functionalization for use in affinity chromatography. Although it has previously been described as an affinity ligand, it is not available either commercially or from the original investigators. The main objective of preparing multiple gram quantities of this compound has been realized, and chromatography columns incorporating this material are being used by Dr. Giri to separate the isozymes of alcohol dehydrogenase from human and other mammalion liver tissues. In addition, a new synthetic route to this compound was developed which has advantages over the literature procedure. The new synthesis produces the desired product in only two steps from commercially available material, and gives an overall yield comparable to the original five step procedure. Some chain-extended analogs are currently being synthesized.

B. Synthesis of 1-acetoxy -4-(2-trimethylammonium-acetyl)-napthalene chloride. November 1984 - February 1985 (ended). In collaboration with I. Kurtz, Laboratory of Kidney and Electrolyte Metabolism, NHLBI, NIH.

The use of "molecular probes" coupled with spectroscopic detection allows the measurement of pH, ion concentrations and potential gradients to be made within the cytoplasm of intact cells. The previously unknown naphthalenic ammonium salt named above was synthesized in four steps from 1-naphthol as part of a search for new types of pH sensitive fluorescent probes. It was hoped that the salt would be concentrated within the cytoplasm due to either active transport through choline channels or passive concentration via the transmembrane potential. Once inside, cellular esterases would reveal the fluorescent phenolate anion (pKa$\sim$8). Although the compound was indeed concentrated and hydrolized in the cytoplasm of tissue cultured cells, the fluorescence of the resulting alkoxide was too weak for use as a pH probe. No further work on this system is anticipated.

C. Analysis of the Pheromone of the Cowpea Weevil. February 1985 - July 1985 (in progress). In collaboration with W. Burkholder. Stored Product and Household Insect Laboratory, USDA, Madison, WI.

The cowpea weevil (Callosobuchus maculatus) is a destructive pest on both the North America and the African continents. It feeds primarily on the cowpea or blackeyed pea (Vigna sinensis). As part of an approach towards pest monitoring and control, the chemical structure of the cowpea pheromone is being sought. Gas chromatography coupled to mass spectral analysis is well suited to the small amounts of volatile materials available to us. Techniques are being developed for running microscale (1 ug) chemical reactions on these samples. Preliminary data are currently being interpreted.

D. Saponins from Alfalfa (Medicago sativa). December 1984 - July 1985 (in progress). In collaboration with D. Guillaume, Faculte de Pharmacie, CNRS, Reims, France.

Alfalfa fodder is known to cause growth inhibition when fed to certain animals (eg. poultry). The active compounds are triterpene saponins. Although the structures of the pentacyclic sapogenin portions of the molecules are known in

some cases, these fragments are inactive without their pendant sugars. Our californium plasma desorption mass spectrometer better allows determination of the molecular weights of such large (MW 1000 1800) and highly involatile compounds. The molecular weights of 21 saponins and saponin derivatives from Alfalfa extracts have been determined so far. The remainder of the structure determinations are being performed by our collaborators in the CNRS.

E. Crystal Structure of a Hydrophobic Cavity Mimic. January 1985 - July 1985 (in progress). In collaboration with J. Silverton, CH, NHLBI, NIH.

Single crystals of a naphthalene cyclophane (for the structure, see J. Am. Chem. Soc. 106: 1492-94, 1984) which were suitable for x-ray Diffraction analysis were grown from pyridine. Inspection of the resulting structure clearly showed the two naphtalene rings separated by three rigid bridges. An analysis of the location of the solvent molecules around the molecular "cleft", which is large enough to engulf a molecule of pyridine, is in progress.

Annual Report of the Clinical Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1985

The research of this Branch is directed toward understanding the underlying causes and developing effective treatment for the major red cell disorders. Red cell disorders that produce significant morbidity and mortality include thalassemia, severe hemoglobinopathies of which sickle cell anemia is the most common, and the various syndromes of bone marrow failure. In our laboratory investigations, we attempt to focus the most advanced techniques and conceptual knowledge on elucidating the pathogenesis and attempting to devise treatments for these disorders.

Patients with either severe beta-thalassemia or sickle cell anemia could benefit from increased production of fetal hemoglobin. During normal human development, HbF ($\alpha_2\gamma_2$) produced in utero, is replaced during the perinatal period with HbA ($\alpha_2\beta_2$). If both beta globin genes are defective either because of mutations that reduce the amount of functional mRNA produced, or because of mutations that result in a structurally abnormal globin (e.g., the β^S globin), the perinatal switch in hemoglobin production leads to the onset of disease. Reactivation of the gamma globin genes in adults could ameliorate the severity of these conditions.

In earlier work by this Branch, 5-azacytidine was shown to stimulate HbF synthesis both in patients with severe beta-thalassemia and those with sickle anemia (see individual project report: "Effect of 5-Azacytidine on Fetal Hemoglobin Synthesis in Patients with Severe Beta Thalassemia and Sickle Cell Anemia"). Detailed analysis suggests that 5-azacytidine affects fetal hemoglobin production both by cytotoxicity to progenitor cells followed by their regeneration and by a direct inductive effect, possibly mediated by its effects on DNA methylation. Concern about the potential carcinogenic effects of 5-azacytidine, evidence that other chemotherapeutic agents such as hydroxyurea and cytosine arabinoside may stimulate HbF synthesis, and the significant but limited increases in HbF have led us to discontinue clinical trials with this agent. Several drugs are being tested singly and in combination in two primate species (baboons and Rhesus monkeys) in an effort to define a regimen that will result in a clinically beneficial increment in HbF (see individual project: "Pharmacological Manipulation of Fetal Hemoglobin Synthesis").

The long term goal of our work is to understand developmental switching in hemoglobin phenotype. This objective can best be realized by gaining insight into the mechanism of gene regulation. Several individual projects are pertinent to these objectives. We are studying the fine structure of the human gamma globin gene promoter to determine whether sequence differences between it and other globin gene promoters are relevant to its selective expression in fetal erythroid cells (see individual project: "Regulation of Hemoglobin Switching During Development: Characterization of the Human Gamma Globin Gene:). Recombinant DNA vectors containing the gamma globin gene and the beta or alpha globin gene have been introduced

into HeLa cells by standard tissue culture methodology. Relative promoter function has been estimated by measuring correctly initiated mRNA transcripts. In such competition experiments, the gamma globin gene promoter is three times more active than that of the beta gene. Human erythroid cells contain two gamma genes and one beta gene on each chromosome 11. In fetal erythroid cells each gene is in a conformation in chromatin that renders it accessible for transcription. Relative promoter "strength" if analogous to that observed in HeLa cells could account for the relative ratio of gamma and beta mRNA produced in fetal erythroblasts.

Truncation and linker scanning mutants have been created and are being studies both in HeLa cells and in an established mouse erythroleukemia cell line. The objective is to define the elements of the gamma globin gene promoter required for efficient transcription and to identify any sequences that account for its relatively high level of function compared to the promoter of the beta globin gene. Using standard recombinant methods, the gamma globin gene promoter has been linked to the coding sequences of a selectable marker that confers resistance to the neomycin analogue, G418. We have shown that a 385 base pair segment of DNA that includes identified promoter sequences is sufficient for selective function of the promoter in a human erythroid cell line. During the next year, we anticipate that this avenue of investigation will allow definition of sequences responsible for selective expression of the gamma compared to the beta gene at various developmental stages.

Cellular differentiation and developmental modulation of gene expression reflects the action of intranuclear proteins that interact with specific regulatory sequences around genes. Such proteins are undoubtedly involved in developmental hemoglobin switching. Proteins that bind selectively to the gamma globin gene promoter are being identified and purified by selective protection of certain DNA sequences after in vitro reconstitution of purified DNA fragments with nuclear extracts (see individual project: Identification of Cis and trans-Acting Factors that Regulate the Human Gamma Globin Gene"). A protein(s) that binds with high affinity 200 base pairs in front of the start site of transcription has been identified in extracts of erythorid and non-erythroid nuclei. A point mutation in this region that causes increased HbF synthesis in humans, also abolishes binding of this protein. This region of DNA is also identified as being of functional relevance by virtue of its hypersensitivity to S_1 nuclease in vitro.

Sequences outside of the gamma globin gene promoter region may also influence developmental expression of the globin genes. Specifically, enhancer sequences that increase promoter function independent of orientation and position with respect to the promoter may be involved in gene regulation. A strategy has been devised for a systematic search for such enhancers. Briefly, cloned segments of DNA are inserted into an expression vector that contains the gamma globin gene promoter linked to the coding sequences of an enzyme readily assayed in cell extracts. A segment downstream from the gamma globin gene region appears to function as an enhancer exclusively in erythroid cells. The exact DNA sequences involved are being defined by creation of appropriate recombinants.

Additional studies pertinent to the interaction of specific proteins with regulatory sequences have utilized the immunoglobulin gene model (see individual project: "Enhancer and Promoter Specificity of Immunoglobulin Genes"). An immunoglobulin gene enhancer and promoter have been linked to the coding sequences of the gene that confers resistance to the neomycin analogue. In separate experiments, we have shown that the immunoglobulin promoter functions selectively in antibody producing cells in a fashion analogous to the selective function of enhancer sequences. An additional selectable marker, the thymidine kinase gene, has been inserted into these vextors and stable transformants that have incorporated the neomycin gene with immunoglobulin gene regulatory elements have been obtained by DNA transfection of mouse L cells. These transformants fail to grow in the neomycin analogue, G418. Activation can be achieved by transfer of the gene for adenovirus E1A protein, apparently by interaction of this protein with the immunoglobulin gene promoter. In analogous experiments, L-cell clones containing the immunoglobulin enhancer-promoter-neomycin resistance gene transcriptional unit serve as recipients for total human or mouse DNA introduced by transfection. Selection for neomycin resistance is used to identify cells that have incorporated genes for proteins that activate either the enhancer or promoter. Several stable transformants have been isolated. Some have arisen by virtue of mutations in the transcriptional complex (cis-activation) whereas other appear to reflect the function of exogenous, newly introduced DNA sequences (trans-activation). The latter are being characterized. Standard gene isolation strategies may be employed to identify the genes responsible for the apparent trans-activation of the immunoglobulin gene control elements.

In our effort to understand the regulation of gene expression, we have also chosen to study a gene that is constitutively expressed in all cells (see individual project: "Characterization of the Gene for Human Dihydrofolate Reductase"). The structure of this gene has been determined and it has been assigned to chromosome 5 using specific molecular probes and somatic cell hybrids. Several intronless pseudogenes have also been characterized and assigned to various human chromosomes. Earlier studies had shown that the promoter region of the function DHFR gene is nucleosome free and hypomethylated whereas the remainder of the gene has the same structural characteristics as transcriptionally inactive chromatin. The structure of this promoter is readily defined by use of cell lines in which the DHFR gene is amplified after selection in the DHFR inhibitor, methytrexate. An 800 bp segment of the promoter is nucleosome-free and the upstream nucleosomes are phased. There are two segments of DNA that are hypersensitive to a variety of endo- and exo-nucleases indicating that they are free of proteins. Separating these two regions is a 200 base pair segment to which is tightly bound two or more protein molecules. Another protein is bound at the transcription initiation site and yet another at the boundary between the nucleosome free region and the upstream phased nucleosomes. Thus multiple proteins are involved in establishing the promoter structure of the DHFR gene in chromatin and these apparently interact with specific DNA sequences. The structure of the DHFR gene is chromatin as defined to date is characteristic of quiescent or non-proliferating cells. Transcription of the DHFR gene is linked to DNA

replication. In planned experiments, we will characterize changes in chromatin structure that occur during DNA replication and transcription of the gene. Methods are also being devised to isolate the proteins that bind to the DHFR promoter region.

Achievement of the differentiated state characteristic of hematopoietic precursors involve selective and coordinated expression of many genes. The cellular analogues of viral oncogenes apparently have important roles in cellular differentiation. Hence we have designed studies (see individual project: "Function of Proto-oncogenes in Human Hematopoietic Cells") to examine expression of these genes in bone marrow cells. The c-fms gene has recently been shown by other investigators to encode for the receptor for monocyte-colony stimulating factor, a hematopoietic regulator required for several aspects of monocyte differentiation and function. We have shown that the human c-fms gene is expressed in human moncytes, both in normal peripheral blood and in the HL-60 leukemia cell line after induction to monocyte differentiation with a phorbal ester. The c-fms gene is deleted from the 5q$^-$ chromosome, present in the bone marrow cells of patients with refractory anemia. Decreased expression of the c-fms gene is apparent on analysis of monocyte RNA from such patients. Furthermore, their erythroid progenitors fail to differentiate in vivo, but can be stimulated to form erythroid colonies in vitro. Monocytes are known to be involved in hematopoiesis. Experiments are being devised to investigate whether defective monocyte function due to deletion of the c-fms gene is responsible for erythroid hypoplasia in patients with 5q$^-$ syndrome.

Another strategy to define the role of certain genes in hematopoietic differentiation involves the introduction of sequences that generate "anti-sense" transcripts complementary to normal mRNA. Formation of intracellular hybrids between mRNA and anti-sense RNA apparently blocks mRNA translation. Recombinant DNA vectors have been constructed in which the MMTV promoter (inducible with dexamethasone) drives the coding sequences of the c-fos or c-myc gene in the sense or anti-sense orientation. Appropriate splice and poly-adenylation-cleavage signals derived from a globin gene are included to allow formation of an appropriately processed transcript. "Sense" and "anti-sense" c-fos vectors have been introduced into 3T3 fibroblasts and human HL-60 cells. Preliminary experiments suggest inhibition of 3T3 cell growth and prevention of differentiation of HL-60 cells that contain the anti-sense transcripts.

Efficient and reproducible transfer of genes into hematopoietic cells may ultimately prove to be useful for functionally correcting the genetic defects in patients with severe hemoglobinopathies, thalassemia, and various enzyme deficiencies. We have explored the use of DNA viruses to achieve gene transfer into normal hematopoietic cells (see individual project: "Use of Viral Regulatory Sequences to Facilitate Gene Transfer and Analysis of Gene Function"). Recombinant adenoviruses have been shown to have the ability to transfer and integrate globin genes into fibroblasts and hematopoietic cell lines in culture but the transformation frequency is inadequate for gene transfer into normal hematopoietic progenitors. In collaborative studies with the Molecular Hematology Laboratory of the NHLBI, we are using recombinant retroviruses for this purpose. A very high

titre amphotrophic recombinant virus containing the neomycin resistance coding sequences has been used to achieve high efficiency gene transfer into a human hematopoietic cell line. A primate model has been devised to study gene transfer in vivo. Adequate nutritional and antibiotic support is provided to sustain the animals through periods of radiation induced pancytopenia prior to reconstitution with autologous bone marrow cells exposed to recombinant retroviruses.

Aplastic anemia reflects the total and catastrophic failure of hematopoiesis. The etiology of aplastic anemia remains obscure. Among the potential causes for this syndrome are viral infection, perturbation of normal cellular interactions essential for effective hematopoiesis, "autoimmune" destruction of hematopoietic stem cells, or an intrinsic abnormality in the stem cell's ability to self-renew. The ability of anti-thymocyte globulin (ATG) to partially or completely restore hematopoiesis in 40-50% of patients with aplastic anemia suggests that many patients have stem cells capable of bone marrow regeneration.

Our studies have identified an activated suppressor T-lymphocyte population in the majority of patients with aplastic anemia (see individual project: "Lymphokines in Aplastic Anemia"). This activated-suppressor population is eliminated following treatment with ATG. These cells are characterized by the presence of HLA-DR and Tac (interleukin-2 receptor) antigens on their surface. We have shown the activated suppressor T-lymphocytes to be of polyclonal origin by analysis of a rearranged T-cell antigen receptor gene using specific molecular probes in Southern blot hybridizations. Thus these cells are likely to be "reactive" to an undefined stimulus. The activated suppressor cells produce gamma-interferon while normal bone marrow contains small amounts of alpha-interferon. Alpha and gamma-interferon act synergistically to inhibit hematopoietic colony formation in vitro. This synergy apparently involves an interacting intermediary cell. A general hypothesis emerges that gamma-interferon production by an activated suppressor population of T-lymphocytes causes bone marrow failure by synergistic action with alpha-interferon in bone marrow. We are attempting to test this hypothesis by treating patients with a monoclonal antibody directed specifically against the Tac positive T-lymphocytes. The available monoclonal antibody was incapable of eliminating the Tac positive population in three patients treated to date. Subsequent patient studies will involve the use of antibody conjugated to pseudomonas toxin in an effort to enhance its ability to abbrogate the target cell population.

The agent that leads to activation of this suppressor population is of great interest. A parvovirus is known to be involved in one form of bone marrow failure, transient erythroblastopenia in patients with hemolytic anemia. The virus infects and destroys erythroid progenitors. Immunity is rapidly acquired and recent experiments have shown no evidence of integration of the viral genome into bone marrow cells of previously infected patients. The role of parvovirus in transient erythroblastopenia have encouraged us to initiate a search for direct evidence of viral infection in the cells of patients with aplastic anemia (see individual project: "Viruses and Bone Marrow Failure"). Extracts of bone marrow

aspirates and extracts of bone marrow cells after co-culture with a variety of tissue culture cell lines have been assayed for reverse transcriptase activity. Two of 25 patients with aplastic anemia consistently show low but definitely positive activity whereas 11 controls were negative. Other evidence for a retrovirus is being sought by electron microscopic examination of fresh and co-cultured bone marrow cells.

Animal models are being developed to further explore the role of viruses in inducing bone marrow failure. Feline parvovirus has been implicated as causing pancytopenia in cats. We have shown that this virus inhibits hematopoietic colony formation in vitro and defining the mechanism of this inhibition. Feline leukemia virus (a retrovirus) is a frequent cause of severe and fatal anemia in cats. Selective inhibition of CFU-E derived colony formation in vitro is caused by this agent. These general experimental approaches should provide insight into the pathogenic mechanisms of viral induced bone marrow failure and may suggest approaches to identifying elusive viruses that act as causitive agents for human disease.

The Clinical Hematology branch has directed a large, multi-center trial of anti-thymocyte globulin (ATG) in aplastic anemia and related bone marrow disorders (see individual project: "Hematopoiesis in Bone Marrow Failure"). More than 150 patients were enrolled. A response rate of approximately 40% has been established. In patients with acute severe disease, two regimens (10 or 28 days of ATG administration) were tested; no significant differences were observed. ATG has been found to be superior to high dose androgen in patients with chronic severe or moderate aplastic anemia. ATG is thought to induce remissions in aplastic anemia by virtue of its ability to destroy lymphocytes. Consistent with this proposed mechanism of action is the observation that a recently produced batch of antilymphocyte globulin (ALG), has been shown to be relatively inactive in the treatment of patients with aplastic anemia. Comparison of this batch to a previous, highly active batch has identified a difference in titer of antibodies directed against several antigens present on T-lymphocytes. These results suggest that effectiveness of ATG or ALG is dependent on very efficient destruction of a pathogenic lymphocyte population.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02208 11 CHB

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Iron Chelation in Transfusional Hemochromatosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

Others: Patricia Griffith, Clinical Nurse Specialist, CHB, NHLBI
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COOPERATING UNITS *(if any)*

Laboratory of Molecular Biology, NHLBI, NIH, Beth., MD
Cleveland General Hospital, Div. of Hematology, Cleveland, Ohio
Cardiology Branch, NHLBI, NIH, Bethesda, MD
Montreal Children's Hospital, Montreal, Quebec, Canada

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies are designed to evaluate the clinical benefits achieved by iron chelation in patients with chronic iron overload. Desferrioxamine is administered by subcutaneous infusion and iron removal is determined by quantitation of urinary iron excretion and careful recording of the total iron administered by transfusion. The results of our 7 year study are summarized in this annual report. Briefly, we found that ascorbic acid given in low dose (3 mg/kg/day) had no significant affects on any measured parameters of iron metabolism nor was there any evident toxicity. Compliance with the chelation regimen was rather poor overall with older individuals in their teenage years showing significantly lower compliance than those in the first decade of life. A relationship between Desferal used and liver iron concentration was substantiated by study of 65 of our patients who had their liver iron concentration measured non-invasively. Efficacy of chelation therapy was not demonstrable in our experience, mainly because individuals with the highest risk for iron overload were among the least compliant in the study. We found that several non-invasive tests used over the past 7 years were not of great value in detecting subclinical iron toxicity. Therefore our followup protocol has been significantly simplified. The study will continue in an effort to document the natural history of individuals with various degrees of compliance with desferrioxamine treatment.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives:

The objectives of these studies are to evaluate iron chelators, to maximize their effectiveness, and to test new chelators as they become available. An effort is being made to develop clinical criteria that will be helpful to determine the efficacy of chronic chelation therapy. A randomized trial of supplemental ascorbic acid was initiated to assess the value and/or toxicity of this agent in promoting mobilization of iron by desferrioxamine in patients with iron overload.

Methods:

Patient populations that participate in these studies include: 1) patients with transfusion dependent congenital or acquired anemia who require regular blood transfusions to sustain life, and 2) patients with idiopathic hemochromatosis at various stages in the process of iron removal by phlebotomy.

Clinical evaluation of organ function include the following:

1) Heart: An estimate of cardiac size by chest-x-ray and electrocardiographic analysis is obtained. Echocardiographic studies are obtained to determine anatomical dimensions of the left ventricle and left ventricular function is assessed by resting ejection fraction. In selected patients, radionuclide angiography is performed at rest and at exercise to determine the functional reserve of the left ventricle.

2) Endocrine evaluation includes specific testing of the pituitary, thyroid, adrenal, pancreatic islets, and gonad function by baseline measurements and various provocative tests.

3) Liver function is determined by standard clinical testing. The liver iron concentration is determined non-invasively based on magnetic susceptibility measurements (N Eng J Med 307:1671-1675, 1983).

4) Serial serum ferritin measurements are obtained to assess the utility of this parameter in estimating total body iron stores and also to follow the course of iron removal.

The following results have been obtained from our studies:

1. Randomized trial of Ascorbic Acid: Forty-seven patients were randomized to receive either ascorbic acid (3 mg/kg/day) or placebo. More than half of the patients complied poorly with the chelation regimen (see below) so that only 11 in the ascorbic acid group and 10 in the placebo group were available for evaluation. The characteristics of the randomized patients on entry into the study are summarized in the following table:

	Ascorbic Acid (11)	Placebo (10)
Age (years)	9.1±3.4	7.8±4.2
Urinary iron Excretion (mg/24hr)	15.3±9.3	13.2±6.9
Ferritin (ng/ml)	3960±2640	3000±1476
Liver Index (SGOT + SGPT)	300±307	224±216
Follow-up Interval (years)	3.8±1.2	3.8±1.0

The following results were obtained by comparing several parameters measured on entry and exit from the study.

	Ascorbic Acid (11)	Placebo (10)
Urinary Iron Excretion (mg/day)	1.26±0.8*	1.3±1.0*
Ferritin (ng/ml)	1.4±1.0	1.0±0.5 (p>0.2)
Liver Index (SGOT + SGPT)	1.4±1.3	0.87±0.64 (p>0.2)

*Fold change end of study value compared to entry

Average Daily Urinary Iron Excretion		
mg/24 hr	16.2±7.1	13.7±9.3

There was no clinical evidence of cardiac disease in either group of patients. Echocardiograms and 24 hours recordings of the heart beat (Holter tracings) remained within normal limits. There were minor changes in thyroid function and glucose tolerance in both groups with no significant differences noted on comparison. One patient randomized to ascorbic acid developed congestive heart failure and died after one and half years although compliance with ascorbic acid had been poor. We concluded that a low dose of ascorbic acid can safely be given to patients on regular subcutaneous deferoxamine although we would recommend that an increase in iron excretion be documented before the patient is prescribed the mediation on a regular bases.

2. Compliance with chelation regimen: The treatment program requires that the patient or parents mix the Desferal, prepare the pump, and initiate the infusion by insertion of a needle into the subcutaneous tissue of the abdomen. The patient must wear the pump 8-12 hours per day. Compliance with this demanding regimen has frequently been less than satisfactory. Age on entry correlated inversely with compliance as the mean age of patients who used the pump 3 days or more per week was 7.9 ± 3.6 years

whereas those who used it 3 days or fewer had a mean age of 12.6 ± 4.9 ($p>0.001$). Young children under the control of their parents comply but older individuals, particularly teenagers, defer from demands of the regimen. This fact has compromised our ability to evaluate the efficacy of chelation in patients at highest risk for symptomatic cardiac disease. Indeed, four poorly chelated older patients died during the study and five other have evidence of significant cardiac disease with high grade ventricular ectopy and/or congestive heart failure.

3. Relationship of Desferal used to liver iron concentration: Two years ago Dr. Gary Brittenham and his colleagues reported the use of a device called a computer-enhanced dual channel super-conducting (SQUID) susceptometer for measuring liver iron concentration noninvasively. Excellent correlation was found between liver iron concentration measured chemically in biopsy specimens and that estimated with the SQUID. Subsequently, Dr. Brittenham studied a small group of patients with iron overload and found that the estimated liver iron concentration was primarily a function of the total number of red cells units given by transfusion during the life-time of the individual and the number of grams of Desferal that the patient had received as part of a chelation regimen. Fifty-five of our patients have had their liver iron concentration measured with the SQUID. The relationship between liver iron concentration, number of transfused units and grams of Desferal administered was also observed in this larger group of patients. These data can be used to predict liver iron concentration over time in individual patients on a regular transfusion schedule who are also treated with one of several chelation regimens. From this analysis, we learned that most patients with significant iron overload will not have appreciable reduction in body iron burden on our standard chelation regimen (1.5 gm of Desferal/day for 5 days per week). Intensive chelation regimens that incorporate periodic intravenous infusions and strategies designed to minimize the total number of units of blood required to maintain a functional hemoglobin must be developed for each patient in an effort to reduce liver iron concentration and total body iron burden.

4. Efficacy of chelation therapy: Several months ago we completed an evaluation of the results of our trial. Twenty-eight patients whose age on entry of the study was greater than 10 years could be separated into chelated (> 3 day/week) or poorly chelated (< 3 day/week) groups. There were 14 patients in the first group and 14 in the second. There was no difference in mean age on entry, urinary iron excretion, serum ferritin, or serum transaminases between the two groups. The average follow-up group for each was 4.5 ± 1.0 years. At the end of this follow-up period there was no significant difference in the ferritin concentration or transaminase levels, the frequency of mild asymptomatic abnormalities of thyroid or pancreatic function, or in the frequency of cardiac function as evaluated by standard noninvasive techniques. Urinary iron excretion at the end of the follow-up period increased an average of 1.6 fold in the poorly chelated group reflecting an increased total body iron burden whereas the urinary iron excretion of the well chelated group did not change significantly during the study. At that time, we concluded there

was no significant difference between the poorly chelated and well chelated groups during the period of observation but noted that there had been little evidence of disease progression in either. Subsequently, several of the poorly chelated patients have developed diabetes or cardiac disease or died. Furthermore, we have not yet completed the evaluation of the relationship between live iron concentration and other indicators of iron overload. This analysis may be more informative since stratification of the patients based on age and chelation compliance is imprecise at best. There is little doubt, however, that a longer period of follow-up will be required to properly evaluate the efficacy of subcutaneous Desferal.

5. Sensitivity of noninvasive tests designed to evaluate the consequences of iron overload: Our previous protocol incorporated extensive noninvasive testing of endocrine and cardiac function in an effort to anticipate significant organ dysfunction prior to the onset of symptoms. Rather minimal progression of disease in the patient population during the period of observation largely obviated the value of this intensive yearly evaluation. Moreover, several parameters such as serum ferritin and urinary iron excretion seem to correlate rather poorly with the total body iron burden although this point will be evaluated more thoroughly during the next year by comparison of recent values to the liver iron concentration measured non-invasively. From our experience over the past six years, there is little doubt that the protocol for follow-up of these patients can be greatly simplified.

Significance to Biomedical Research and to Institute Program:

Hemosiderosis is a major cause of morbidity and mortality in patients requiring prolonged transfusion therapy. The role of iron chelators in improving the clinical course of these patients must be ascertained.

Proposed Course of the Project:

This project will continue until a suitable iron chelator is found and evaluated or until the need for transfusion therapy in thalassemia and other congenital hemolytic anemias is removed. Chelation treatment seems imperative as new advances in genetic techniques offer increasing promise that gene therapy may ultimately be feasible. We recommend aggressive chelation appropriate to the patient's degree of iron overload and evidence of cardiac or endocrine toxicity. Two patients have been started on continuous intravenous desferrioxamine self-administered through a Hickman catheter. From careful follow-up of the entire patient population a judgment regarding efficacy should become feasible.

Publication:

Nienhuis, A.W.: The Thalassemias. In Wyngarden, J.B. and Smith, L.H., Jr. (eds.), Cecil Textbook of Medicine, 17th Edition, W.B. Saunders, Philadelphia, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02307 06 CHB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)* Use of Viral Regulatory Sequences to Facilitate Gene Transfer and Analysis of Gene Function

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Stefan Karlsson, M.D., Ph.D., Visiting Associate, CHB, NHLBI

Others Stefan Schweiger, B.S., Research Assistant, CHB, NHLBI
Amanda Cline, Research Assistant, CHB, NHLBI
Austine Moulton, Research Assistant, CHB, NHLBI
Arthur Nienhuis, M.D., Branch Chief,, CHB, NHLBI
Yakov Gluzman, Ph.D., Cold Spring Harbor Laboratory, New York
Kevin Van Doren, Ph.D., Cold Spring Harbor Laboratory, New York

COOPERATING UNITS *(if any)*

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

LAB/BRANCH

Clinical Hematology Branch

SECTION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The immediate goal of these studies is to develop methods for efficiently introducing human globin genes into hemopoietic cells both from tissue culture lines and normal bone marrow to study their tissue specific regulation. A hybrid SV 40 virus which contains the prokaryotic gene coding for chloramphenicol acetyl transferase (CAT) has been constructed and used to transfer, and transiently express the CAT gene in hemopoietic cell lines and fresh bone marrow cells of humans and other species. Recombinant SV 40 lysates contain wild type SV40 virus which severely compromises their use to introduce genes permanently into cells and cell lines. We have therefore made use of helper free recombinant adenoviruses that contain the neomycin resistant gene (neo) and have successfully transformed the hemopoietic cell lines K562 and MEL to G418 resistance. We have also constructed double recombinant adenoviruses containing the neo gene and the human beta globin gene. This virus can successfully transfer both genes into K562 and other cell lines. Although the transformation frequency of adenoviral vectors is considerably higher than that of DNA mediated transfer, it has not proven to be high enough for hemopoietic stem cells. Therefore retroviral vectors containing a human globin gene and a selectable marker gene are being constructed to achieve gene transfer into hematopoietic stem cells in vitro and in vivo.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives:

The goal of correcting human genetic defects which result in severe disease by genetic therapy is becoming an increasingly more realistic objective based on substantial knowledge about gene structure and function derived from exploitation of the molecular cloning and recombinant DNA technology. Much remains to be learned about the specific regulatory sequences which enable the high level expression of globin genes in a stage and tissue specific manner. Needed are efficient methods for introducing globin genes into hemopoietic cell lines. $CaPO_4$ mediated DNA transfer techiques are very inefficient for cells which grow in suspension and for nontransformed normal cells. We have successfully used an SV 40 recombinant containing the chloramphenicol acetyl transferase (CAT) gene to introduce the CAT gene into various hemopoietic and other cell lines as well as bone marrow cells from mouse, monkey and man. An SV 40 recombinant containing the DHFR methotrexate resistant gene has successfully been used to transform CHO DG21 cells to methotrexate resistance. Furthermore, adenoviral recombinants that contain the neomycin resistance gene have successfully been used to transform both K562 and MEL cells to G418 resistance. The adenovrius can easily carry two genes separated by 20 kilobases of relatively inactive DNA and is therefore an ideal viral vector to introduce two genes, e.g. a human globin gene and a selectable marker at the same time. The human beta globin gene has now been transferred into the human erythroleukemia cell line K562 and other cells lines. We have also used a retrovirus vector containing the neomycin resistance gene to transfer K562 cells to neomycin resistance and are currently using the same vector to transform hemopoietic stem cells in vitro (human) and in vivo (monkey). As retroviruses have very high transformation frequency we are currently constructing recombinant retroviruses containing both a selectable marker gene and human globin gene. These vectors will be used to transfer globin genes stably into hemopoietic cell lines and also bone marrow stem cells from monkey and man.

Methods:

1. Adenoviral recombinants that lack the E1 gene, including the transforming E1a gene and also a part of the E3 gene are constructed by placing the insert either in the E1 or E3 region of the virus. This can be accomplished either by in vitro ligation of the insert and the two viral arms followed by transfection into 293 cells, or the construction can be done by recombination then by cotranfecting into 293 cells two viral fragments (one containing the insert) with sufficient overlapping sequences to allow recombination. 293 cells that express the E1a protein can replicate the otherwise replication defective recombinant virus and a helper free pure recombinant virus can thereafter be plaque purified, grown up and analyzed by restriction enzyme analysis and Southern blotting.

2. To construct recombinant retroviruses plasmids containing recombinant retroviral genomes have been obtained from other laboratories. They contain

either the neomycin resistance gene or the methotrexate resistant dyhydrofolate reductase (DHFR MtxR) gene. These plasmid vectors usually contain a single restriction endonuclease cutting site within the retroviral genome, allowing the insertion of another gene into the retroviral part of the plasmid. Using standard molecular biology techniques double recombinant retroviruses can be constructed containing a selectable marker gene (neo or DHFR) and the gene of interest for example a globin gene. To make a helper free recombinant retrovirus the retroviral packaging cell lines, psi two (2) and PA-12 are utilized. Psi two is a mouse 3T3 cell line that has been transformed with a defective retroviral genome. This cannot in itself be packaged into a viral particle but can produce all the proteins needed for packaging of retroviruses. The PA12 cell line is an analogous cell transformed with a defective amphotropic retrovirus and can be used to produce amphotropic recombinant retroviruses that will be helper free. The retroviral plasmids are transfected into the psi two cell line and the cells are selected in G418 if the retrovirus contains the neo gene or methotrexate if the retrovirus contains the DHFR MtxR gene. The surviving cell clones are thereafter grown up and used to produce a helper free ecotropic retrovirus. The retrovirus in the cell supernatant ia then used to transform PA-12 cells to create a recombinant amphotrophic retrovirus. Transformed cell clones are again identified using selective medium. Transformed cells are grown up, DNA from them is extracted and analyzed by Southern blotting to verify presence of unrearranged DNA inserts in the retrovirus. The viruses are thereafter titered on 3T3 cells.

3. Transformation of tissue culture or bone marrow cells: Cell lysate from the adenovirus producing 293 cells or purified adenoviruses which have been separated on cesium equilibrium gradient are used to infect cell lines. The retroviral infections are done using supernatant from the virus producing cells to infect hemopoietic cell lines or fresh bone marrow cells. The cells to be transformed by retrovirus can also be co-cultured with the virus producing cells. After the infection period cell lines are incubated in vitro with selection medium to identify the cells that have been transformed. For gene transfer into monkeys approximately 10^9 bone marrow cells are harvested from the pelvis and femor of rhesus monkeys and the marrow cells separated on a percoll gradient. The mononuclear cells are thereafter infected with retrovirus either by supernatant or by co-culture with the virus producing cells. The infection time is from 2-24 hours with or without agents that stimulate cell division. After radiation of the animal the infected bone marrow cells are injected into the animals which all have a central venous catheter to facilitate fluid and nutrition therapy. During the aplastic period the monkeys are hyperalimented with Dextrose and aminoacids. Broad spectrum antibiotics are also given. Blood and platelet transfusions are given as needed. When the monkey reconstitutes 3-4 weeks after radiation cells from peripheral blood are harvested and their DNAs analyzed by Southern blotting.

4. Analysis of globin gene expression. RNA is harvested for 5' mapping using probes specific for globin messenger RNA sequences, using the S1 nuclease mapping technique.

Results:

1. A double recombinant adenovirus containing the human beta globin gene in the E1 region and the neomycin resistance gene in the E3 region has been constructed and the lysates of this virus has been used to transform fibroblasts from human and hamster and also K562 and MEL cells. The transformation frequency of fibroblasts and K562 cells in high but the transformation of MEL cells is lower or about 10^{-5}. DNA has been extracted from the cells and analyzed by the Southern blotting technqiue and shown to contain both the human beta globin gene and the neomycin resistance gene except in the MEL cells where the human beta globin gene has not been detected. Only a small portion of the viral genome intergrates into the MEL cell clones analyzed so far but all other cells analyzed have intergrated the whole adenoviral genome.

2. A recombinant adenovirus containing the methotrexate resistant DHFR gene driven by the long terminal repeat from Rous sarcoma virus has been constructed. The inserted gene is in the E1 region of the recombinant virus. This virus has been shown to contain the intact inserted gene by Southern blotting of purified viral DNA. However, it has been difficult to use it to transform fibroblasts cell lines to methotrexate resistance. The transcription unit of the inserted gene does not work well in this virus for reasons that remain unclear.

3. Expression of the human beta globin gene in the double recombinant adenovirus containing the human beta globin gene and the neomycin resistance gene (adeno human beta neo) has been studied in 293 cells. 293 cells replicate this virus to a high copy number. RNA has been extracted from the cells and analyzed by S1 mapping. Correctly initiated RNA was demonstrated but protection from S1 nuclease cutting of the full length probe was also seen. This may indicate some upstream initiation perhaps by the E1a promoter which is situated upstream from the human beta globin gene in the recombinant virus. S1 mapping experiments using RNA isolated from fibroblasts and K562 cells transformed with this adeno-human-beta-neo virus has not detected any human beta globin RNA. Collectively these experiments indicate that the human beta globin gene in the double recombinant virus is intact, functional and correctly regulated in the cell lines it has been tested in.

4. A plasmid (pZipNeo) containing a recombinant retroviral genome with the neomycin resistance gene driven by the SV 40 promoter was obtained from Dr. Mulligan's laboratory. This plasmid was used to create an amphotropic retrovirus by transfection into psi 2 cells and using the ecotropic virus to infect PA-12 cells and thereby create an amphotropic virus, with titer of 10^6 cfu per ml. This virus was used to transform MEL cells to neomycin resistance. Another amphotrophic retrovirus, XM5 created in Dr. Anderson's laboratory has been used to transform K562 cells to neomycin resistance. The latter virus has also been used to infect bone marrow cells from monkey in an attempt to transfer the neomycin resistance gene into the hemopoietic stem cells of the animal.

Significance to Biomedical Research in the Institute Program

Many serious human diseases arise because of monogenetic mutations affecting the strucural or enzymatic component within cells. Two of these in which we are particularly interested are sickle cell anemia and homozygous thalassemia. The goal of achieving genetic therapy for these condtions should be obtainable with increased knowledge of globin gene regulation and the development of gene transfer methodology. In the course of pursuing this objective additional knowledge will be gained regarding the function of transcriptional regulatory signals in eukaryotic cells.

Proposed Course of the Project

1. The adeno human beta neo recombinant virus has been used to transform MEL cells with the aim to transfer the human beta globin gene into these cells and obtain expression of the human globin gene, since MEL cells are know to express the beta globin gene. So far we have not been able to transfer the human beta globin gene into MEL cells but by uaing a purified form of the virus we have now been able to create a large number of MEL cell clones some of which may include the human beta globin gene. Simultaneously we are making hybrids between MEL on one hand and on the other hand two human cell lines, K562 and VA2 fibroblasts, that have been transformed with the adeno-human-beta-neo virus. This should lead to hybrids containing a human chromosome with the viral genome intergrated but at the same time containing all the transacting regulatory elements to allow expression of the transfered human beta globin gene.

2. A new double recombinant adenovirus is being constructed containing a hybrid human globin gene consisting of the 5' end of the gamma gene and 3' end of the beta gene in the E1 region of the adenovirus and the neomycin resistance gene in the E3 region of the adenovirus. The hybrid globin gene in this virus should behave similar to the gamma globin gene which is known to be expressed in K562 cells. Since recombinant adenoviruses are know to transform K562 cells readily and our previous studies have shown that the full adenoviral genome intergrates into the K562 chromosomes, the gamma beta hybrid gene should be expressed in the K562 cell line.

3. Experiments are currently ongoing using recombinant retroviruses to transfer genes into the bone marrow stem cells of rhesus monkeys. The first virus, XM5 that has been used contains only the neomycin resistance gene which cannot be selected for in vivo. Viruses are now being prepared containing the methotrexate resiatant DHFR gene to be used in subsequent experiments in order to be able to amplify the cell population that has been transformed with the retrovirus using methotrexate (in vivo selection). Currently only the highest titer recombinant retroviruses are being used for these experiments and if successful more complex recombinant retroviruses containing a selectable marker gene and another gene for example a globin gene will be constructed and used.

4. Construction of more complex retroviruses containing globin genes, alpha 1 antitrypsin, etc. are now in progress using previously mentioned viral vectors pZipneo and XM5 as recipient vectors. The resulting viruses should therefore contain a selectable marker gene plus a globin gene or another gene of interest. Previous data shows that retroviruses can be used to transform hemopoietic cell lines MEL and K562. These double retroviruses can therefore be used to atudy globin gene regulation in these cell lines. If viruses of sufficiently high titer will be produced transformation of bone marrow stem cells will also be attempted using these viruses.

Publications:

1. Karlsson, S., Humphries, R.K., Gluzman, Y., Nienhuis, A.W.: Transfer of gene into hematopoietic cells using recombinant DNA viruses. Proc. Natl. Acad. Sci USA 82: 158-162, 1985.

2. Humphries, R.K., Karlsson, S., Nienhuis, A.W.: Toward Treatment of Genetic Anemias by Gene Replacements. In Principles and Techniques of Human Research and Therapeutics. Mt. Kisco, New York, Futura, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02310 05 CHB

PERIOD COVERED
October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of the Gene Human Dihydrofolate Reductase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboratory, and institute affiliation)*
Takashi Shimada, M.D., Visiting Associate, CHB, NHLBI
Others: Koiti Inokuchi, M.D., Visiting Fellow, CHB, NHLBI
Henry Lin, M.D., Medical Staff Fellow, CHB, NHLBI
Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

COOPERATING UNITS *(if any)*
Clinical Hematology Branch

LAB/BRANCH
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The chromatin structure of the promoter region of the human DHFR gene was studied using DNaseI, micrococcal nuclease, exonuclease III, Bal 31 and various restriction enzymes. Mapping of endonuclease sensitive sites and an exonuclease protection assay showed that the DNA fragment between -670 and +150 base pairs (bp)from the initiation site of the DHFR gene is nucleosome free. Five discrete DNaseI hypersensitive sites were mapped in this region. A 170 bp fragment that extends from -340 to -170 was shown to be resistant to both endo- and exonucleases, suggesting that multiple proteins are bound to this region. Another protein binding site was mapped at the initiation site of the gene. DNA mediated gene transfer using a series of deletion mutants of a DHFR minigene showed that a minimal promoter segment containing a TATA box is sufficient for gene expression. Methylation of the DHFR minigene in vitro reduced transforming frequency to 10-20% of control. In cells transformed with the methylated gene only a region corresponding to the initiation site was specifically demethylated. The DHFR minigene was found to be amplified up to 500 fold in CHO cells. Amplification occured within a few weeks after transfection in the absence of MTX, and the amplified genes were maintained stably without selection.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives:

One objective of this study is to compare the mode of regulation of constitutively expressed genes to the mechanism of regulation of the genes that encode for products characteristic of the differentiated state. Constitutively expressed genes are generally crucial for cell proliferation and growth while specialized genes are most relevant to development of the differentiated state. Dihydrofolate reductase (DHFR) was chosen for study because of its essential role in cell metabolism; it is required for the synthesis of deoxynucleotide precursors utilized for DNA synthesis.

The second objective of these studies is to characterize amplification of the DHFR gene. Growth of cultured cells in methotrexate, a selective inhibitor of dihydrofolate reductase, often leads to amplification of the DHFR gene. Our purpose is to compare the structure and organization of functional DHFR genes in normal and methotrexate resistance cells and thereby to deduce certain features relevant to the mechanism of gene amplification.

We have previously cloned and characterized the functional human DHFR gene and three intronless DHFR pseudogenes. These four genes are dispersed in that each one is found on a different chromosome. The functional gene has been assigned to chromosome 5, and three pseudogenes, hDHFR 1, 2 and 4 to chromosome 18, 6, and 3, respectively. The hDHFR 1 has perfect sequence homology to the functional locus; it was found to be polymorphic in that it is present in DNA from some individuals but not others. The DHFR 3 contains only exons 4 to 6. A member of the Alu moderately repetitive DNA sequence family has also been inserted into this chromosomal site resulting in interruption of the DHFR coding sequence. The functional gene is about 30 kb long and consists of six exons and five introns. A single transcriptional start site and three polyadenylation sites have been identified; these account for the 0.8, 1.0, and 3.8 kilo nucleotide DHFR mRNA species in human cells. A major transcript has an exceptionally long 3' end untranslated region extending for 3 kb.

The sequence of the 5' promoter region is highly homologous to the corresponding region of the mouse DHFR gene; it is is extremely rich in guanosine and cytosine residues. Only this promoter region was found to be undermethylated, while the reamining 30 kb gene was completely methylated. Only the promoter region was DNaseI hypersensitive in chromatin. In MTX resistant HeLa cells with an amplified DHFR gene, all copies of the amplified gene exhibited a pattern of undermethylation and DNaseI hypersensitivity of only the promoter region.

We have now characterized the structure and function of the constitutively active promoter by detailed mapping of sequences in the promoter region onto which regulatory proteins may be bound. In a second approach, the promoter region of the DHFR minigene was modified by either in vitro methylation or deleting various sequences in the 5' flanking region. Using

a stable transformation assay, system we are studying the function of these modified promoters in DHFR CHO cells.

Methods:

1. Mapping of endonuclease sensitive sites: Isolated nuclei from MTX-resistant HeLa cells were digested with increasing amounts of a variety of endonucleases including DNaseI, micrococcal nuclease and various restriction endonucleases. DNA was purified, restricted with other enzymes and analyzed by Southern blot hybridization (indirect end labeling technique).

2. Exonuclease protection assay: Isolated nuclei were digested with a restriction enzyme and variable amounts of one of the exonucleases, exonuclease III or Bal 31. Exonucleases can digest along the DNA from the free end made by restriction enzyme digestion until the nuclease encounters a specific block due to binding of nuclear proteins. The position of the blocked sites were determined by the indirect end labeling methods after Southern blots were made.

3. Plasmid constructions: The human DHFR minigene was constructed by recombining the 5' end of the functional gene with the coding and 3' end of the hDHFR 1 gene, a previously characterized perfect intronless pseudogene. A series of deletion mutants of the DHFR minigene was constructed by removing various restriction fragments from the promoter region and rejoining the rest of the minigene. The 3' end of the normal gene was substituted for that of the pseudogene.

4. Methylation pattern of the transfected DHFR minigene: The DHFR minigene was methylated in vitro with either bacterial HpaII methylase or HhaI methylase in the presence of S-adenosyl methionine. DHFR CHO cells were transfected with the methylated DNA and selected for the DHFR$^+$ phenotype. DNA was extracted from the transformed cells and the methylation pattern of the integrated DHFR gene was assayed using the methylation sensitive restriction endonucleases, HpaII, MspI or Hha I.

Major Findings

1. The promoter region of the DHFR gene was shown to be highly sensitive to a variety of endonucleases. Two regions from -600 to -350 and from -150 to +100 from the Cap site were sensitive to DNaseI but a 200 bp fragment in between the two DNase I sensitive region was highly resistant to DNaseI. Detailed mapping of the DNaseI hypersensitive region revealed five discrete cutting sites in these regions. Restriction endonuclease cutting sites within the DnaseI hypersensitive regions were sensitive, while the sites in the DNaseI resistant region were protected in chromatin.

2. The promoter region is nucleosome free. DNA purified from micrococcal nuclease treated nuclei was blot hybridized to various probes. When the blot was hybridized to the coding sequence, the 5' flanking, or the

intron I probes a typical nucleosomal ladder pattern was observed, but no distinct ladder was seen on hybridization with the promoter probe that includes the DNaseI hypersensitive region. Thus, the normal nucleosome array of the DHFR gene is interrupted at the promoter region by about 800 bp of nucleosome free DNA.

3. Nucleosomes are phased in the 5' flanking region adjacent to the promoter region. Both sides of the nucleosome free promoter region were generally insensitive to endonucleases except for micrococcal sensitive sites located at about 200 bp intervals. In between these sensitive sites, several preferential micrococcal cutting sites were mapped in purified DNA. However, these sites were highly protected in chromatin probably due to binding of nucleosomes.

4. Multiple nuclear proteins bind to the promoter region. The protein binding sites in the nucleosome free promoter region were determined using an exonuclease protection assay. Multiple barriers against exonucleases were detected between -340 and -170 indicating binding of a protein complex composed of multiple molecules. This complex is not dissociated by high salt concentrations. Another protein binding site was mapped at the initiation site of the gene.

5. The DHFR promoter is enhancer independent and a minimal promoter containing a TATA box is sufficient for gene expression. A DHFR minigene without an SV40 enhancer transformed DHFR$^-$ CHO cells at a significant frequency, although the transformation was 5 to 10 times less than that of the gene with the SV40 enhancer. Transfection experiments using a series of 5' flanking deletion mutants of the DHFR minigene showed that the minimal promoter containing only 72 bp of upstream sequence is sufficient for gene expression. The minimal promoter was linked to the bacterial chloramphenicol acetyl transferase gene or the gene conferring neomycin resistance. Both test genes were active in mouse L cells.

6. The DHFR minigene requires the 3' flanking sequence and a minimal intron length for efficient transcription. Because the 3' end region of our original DHFR minigene was composed of an intronless gene, the sequence downstream from the polyadenylation site is different from that of the functional DHFR gene. The minigene has an A rich tract at the 3' end and the sequences downstream are derived from the DNA into which the intronless gene was inserted. We made a new DHFR minigene that has the same 3' end flanking sequence as the functional gene. This new plasmid gave rise to DHFR$^+$ colonies with a 10 times higher frequency. No colonies appeared when cells were transfected with an intron deletion mutant that lacks a 250 bp fragment between 57 bp downstream from the 3' end of Exon 1 and 50 bp upstream from the 3' end of the intron 1. However, inserting a 200 bp pBR322 fragment into the intron deletion mutant restored activity of the minigene. Thus no specific internal intron sequences appeared to be needed but a minimal intron length is important.

7. Undermethylation at the initiation site appears to be essential for gene activation. The DHFR minigene that was methylated in vitro was only 10-20% as efficient in transforming DHFR$^-$CHO cells as the unmethylated gene. Only a small region of the gene immediately upstream from the start site of transcription became undermethylated on transformation to the DHFR$^+$ phenotype. The nucleotides upstream and downstream, normally undermethylated in cells containing the endogenous gene, remain completely methylated in the CHO transformants.

8. The promoter region of the DHFR minigene introducted by DNA transfection, has similar DNaseI hypersensitivity as the endogenous gene. The exonuclease protection assay showed that one of the binding proteins at -270 bp was not present. This molecule seems to be tissue or species specific. The same DNase hypersensitivity and protein binding pattern were observed in the promoter region of the highly methylated DHFR minigene.

9. The DHFR minigene was amplified in CHO cells in the absence of MTX. During the course of transfection experiments, we found that the human DHFR minigene was amplified up to 500 fold within a few weeks in a selective medium for DHFRgene expression. This rapid gene amplification is probably due to inefficient transcription of the minigene such that multiple DHFR gene-copies are required to produce sufficient enzyme for the cells to survive.

Significance to Biomedical Research and the Institute Program:

This project is designed to increase our understanding of transcriptional signals that operate at the DNA sequence level in modulating gene expression. The phenomenon of gene amplification, if understood and applied, might have important implications for genetic engineering of eukaryotic cells. Furthermore, the DHFR gene is one of the few available potential dominant selectable markers for use in gene transfer into eukaryotic cells.

Proposed Course:

Recent study of the mouse DHFR gene showed the DHFR gene is cell cycle regulated at the transcriptional level. Thus, we are interested in changes in chromatin structure of the DHFR gene during the cell cycle. Using CHO cells transfected with deletion mutants of the DHFR minigene, we will determine which region of the promoter is responsible for cell cycle regulation. Isolation and characterization of the promoter binding proteins will also be attempted.

Various tissue specific inducible genes including globin, immunoglobulin and interferron genes will be inserted into the plasmid containing the DHFR minigene. These plasmids will be introduced into various human cell lines. Cells containing multiple copies of the DHFR minigene will be selected by

exposure to increasing concentrations of MTX. Multiple copies of the co-amplified tissue specific gene will allow us to study their regulation and chromatin structure.

Publications:

Anagnou, N.P., O'Brien, S.J., Shimada, T., Nash, W.G., Chen, M.-J., and Nienhuis, A.W.: Chromosomal organization of the human dihydrofolate reductase genes: Dispersion, selective amplification and polymorphism. Proc. Natl. Acad. Sci. USA. 81:5170-5174, 1984.

Shimada, T., Chen, M.-J., and Nienhuis, A.W.: A human dihydrofolate reductase intronless pseudogene with an Alu repetitive sequence: Multiple DNA insertions at a single chromosomal site. Gene. 31:1-8, 1984.

Shimada, T., Nienhuis, A.W.: Only the promoter region of the constitutively expressed normal and amplified human dihydrofolate reductase gene is DNaseI hypersensitive and undermethylated. J. Biol. Chem. 260: 2468-2474, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02312 04 CHB

PERIOD COVERED
October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effect of 5-Azacytidine on Fetal Hemoglobin Synthesis in Patients with Thalassemia and Sickle Cell Anemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
Timothy J. Ley, M.D., Senior Investigator, CHB, NHLBI
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R. Keith Humphries, M.D., Visiting Scientist, CHB, NHLBI
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COOPERATING UNITS *(if any)*
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LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

5-Azacytidine, a cytidine analog, causes an induction of fetal hemoglobin synthesis in patients with thalassemia or sickle cell anemia. The mechanism of this effect is not yet clear. Two major hypotheses have dominated our experiments in this area: 1. that 5-azacytidine, in inducing genomic DNA hypomethylation, directly reactivates the gamma globin genes, and 2. that by disturbing the normal maturation of erythroid progenitor cells, the fetal program was somehow reactivated, allowing the progeny of these stem cells to produce more fetal hemoglobin. The available data suggest that the drug may act via a combination of hypomethylation and other "inductive" effects whose molecular mechanisms are not understood. Because of the potential carcinogenicity of this drug when administered chronically to humans, and the only moderate increase in HbF observed, we have discontinued human trials with 5-azacytidine. Our experiments regarding the mechanism of the drug are completed. For this reason, this project is being terminated.

PHS 6040 (Rev. 1/84) GPO 914-918

Significance to Biomedical Research and to Institute Program:

The work described above and previous studies have clearly shown that it is possible to augment fetal hemoglobin synthesis in adults with hemoglobinopathies. This basic finding has spawned a number of other studies designed to evaluate the mechanism of the phenomenon, and to further investigate possibilities for clinical applications. Our basic observations have lent considerable insight into mechanisms of gene regulation in vivo, and have suggested ways by which fetal hemoglobin production might be augmented using other cytotoxic drugs.

Publications:

Chiang, Y. L., Ley, T.J., Sanders-Haigh, L., and Anderson, W.F.: Human globin gene expression in hybrid 2S MEL x human fibroblast cells. Somatic Cell and Molecular Genetics 10:399-407, 1984.

Ley, T.J., Chiang, Y., Haidaris, D., Anagnou, N.P., Wilson, V., and Anderson, W.F.: DNA methylation and regulation of the human -like globin genes in mouse erythroleukemia cells containing human chromosome 11. Proc. Natl. Acad. Sci. USA 81:6618-6622, 1984.

Humphries, R.K., Dover, G., Young, N.S., Moore, J.G., Charache, S., Ley, T.J. and Nienhuis, A.W.: 5-Azacytidine acts directly on erythroid precursors and progenitors to increase production of fetal hemoglobin. J. Clin. Invest. 75:547-557, 1985.

Sands, J.M., Macher, A.M., Ley, T.J. and Nienhuis, A.W.: Disseminated infection caused by Cunninghamella bertholletiae in a patient with thalassemia. Ann. Int. Med. 102:59-63, 1985.

Ley, T.J. and Nienhuis, A.W.: Induction of hemoglobin F synthesis in patients with thalassemia. Ann. Rev. Med. 36:485-498, 1985.

Ley, T.J. and Nienhuis, A.W.: Prospects for therapeutic manipulation of human gene function. In Principles and Techniques of Human Experimentation. Futura Press, Futura, New York, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02313 03 CHB

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Hemoglobin Switching During Development: Characterization of the Human Gamma Globin Gene.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Principal Investigator: Nicholas P. Anagnou, M.D., Guest Worker, CHB, NHLBI

Others: Tim Rutherford, Ph.D., Visiting Associate, CHB, NHLBI
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Stefan Karlsson, M.D., Visiting Associate, CHB, NHLBI
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Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to determine whether DNA sequence differences in the promoter regions of the several human globin genes are relevant to their developmental regulation. In the past, our studies have focused on the gamma globin gene promoter. In experiments that utilize recombinant DNA vectors in which the gamma globin gene promoter is linked to the coding sequences for neomycin resistance marker, we have shown that 385 bp of sequence are sufficient for erythroid specific expression as tested by stable transformation of various cell lines. Linker scanning mutants in which a synthetic linker replaces portions of the promoter have been constructed so as to delete and/or replace conserved sequences within the promoter region. Studies utilizing these mutant promoters have shown that the duplicated "CCAAT" segment, the feature by which the gamma promoter differs from that of the beta globin gene, is not required for promoter function in either erythroid or non-erythroid cells. Hybrid promoters containing portions of the gamma and beta promoter regions are being constructed in an effort to define those sequences that determine developmentally specific regulation of the gamma globin gene. These experiments utilize the human erythroleukemia cell line, K562, in which the gamma globin gene but not the beta globin gene is expressed.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives:

During erythroid differentiation in humans, there is a specific pattern of globin gene expression; embryonic hemoglobins are produced during the earliest stages of gestation, fetal hemoglobin during the last two trimesters, and adult hemoglobin after birth. The human gamma and beta-globin genes are expressed during the fetal and adult developmental periods, respectively. Differences in the sequences in the promoter region of these genes may be relevant to their developmental regulation. There is accumulating evidence that coordinate expression of individual globin genes may be mediated by trans-acting regulatory factors produced during the various stages of development (embryonic, fetal, and adult) and specific for particular genes. These trans-acting factors apparently interact with cis-specific sequences within or flanking the regulated genes thus modifying their expression sequentially during ontogeny.

The objectives of this project are to understand the molecular basis of hemoglobin switching during development. This process is of fundamental clinical importance since activation of the dormant gamma globin gene in individuals with severe beta thalassemia and sickle cell anemia has been shown to be of clinical relevance (see individual project: "Effect of 5-Azacytidine on Fetal Hemoglobin Synthesis in Patients with Severe Beta Thalassemia and Sickle Cell Anemia"). The questions addressed are as follow. First, are the sequence differences demonstrated between the promoters of the individual globin genes relevant to developmental switching? Second, what portion of the gamma globin gene promoter is required for selective expression in cells producing HbF? Third, can strategies be devised to isolate genes that encode proteins involved in gamma globin gene activation?

Methods:

1. Recombinant DNA molecules containing the gamma, beta, or alpha globin gene and a SV-40 enhancer sequence were constructed. These were introduced into HeLa cells. After 48 hours RNA was extracted and analyzed to determine the relative concentration of mRNA transcripts from the various globin genes. A sensitive S_1 nuclease assay was used.

2. The same vectors were modified by removal of the SV-40 enhancer sequence and by insertion of a transcriptional unit containing the coding sequences for a gene that confers neomycin resistance to eukaryotic cells. These modified vectors were introduced into mouse erythroleukemia cells and stably transformed clones were identified and characterized. Integrated DNA sequences were mapped by Southern blotting and RNA, extracted from cells before and after induction to erythroid maturation, was analyzed by the S_1 nuclease method.

3. A human ^{G}gamma promoter extending from 385 base pairs (bp) upstream of the transcriptional start site down to the middle of the 5' non-coding

region was joined to the neomycin resistance gene by a Hind III linker and cloned into pBR322 ("Gamma-neo Construct"). Other promoters were similarly joined to the neo gene. SV-40 and MaSV enhancers were cloned into an Eco RI site immediately 5' of the promoter. A putative tissue specific enhancer located downstream from the $^{A}\gamma$ gamma globin gene was similarly cloned into this site. These constructs were transfected into the human erythroleukemia cell line, K562, by electroporation. Cells were cloned in methylcellulose medium containing G418 and macroscopic colonies scored by visual inspection three weeks later. Promoter efficiency was measured by the number of G418 resistant colonies formed, relative to the number formed after transfection with a control vector in which the neo coding sequences were under the control of a SV-40 promoter.

4. Truncation and linker scanning mutants of the gamma globin gene promoter have previously been constructed by standard recombinant DNA techniques (see previous individual project reports). During the past year, these mutants have been introduced into mouse erythroleukemia cells and stabily transformed clones have been identified and characterized.

5. Hybrid promoters containing elements from the gamma and beta globin gene promoter regions are being constructed by standard recombinant DNA techniques. These hybrid promoters are introduced into K562 erythroleukemia cells by DNA transfection or electroporation. Stabily transformed clones are identified and the relative level of mRNA transcribed from genes driven by the hybrid promoters is determined by S_1 nuclease analysis.

Major Findings:

1. Relative strengths of the gamma and beta promoters: Cotransfection of vectors containing either the alpha and beta or gamma globin genes into HeLa cells revealed the gamma promoter to be stronger than that of the beta gene. The relative ratio of transcription from these genes was approximately two-fold. Direct competition between the gamma and beta promoters was achieved by cotransfection of vectors containing these genes. In such experiments, the gamma promoter was three-fold more active than that of the beta globin gene.

2. Sequences required for optimal expression of the gamma globin gene promoter: Deletion of sequences beyond 260 bp upstream from the gamma globin gene transcriptional start site did not decrease promoter function in either HeLa or mouse erythroid cells. Deletion of one of the segments containing the duplicated conserved sequence ("CCAAT") similarly did not decrease but actually increased gamma gene expression in both erythroid and non-erythroid cells. These mutants are being introduced into human erythroleukemia cells in which the gamma but not the beta globin gene is expressed to determine whether the removed sequences are involved in developmentally stage specific expression of the gamma globin gene.

3. $^{A}\gamma^{G}$ gamma globin promoter truncated 385 bp upstream of the transcriptional start site drives the neo gene more efficiently in K562

cells than the strong viral promoter of the pSV2 neo vector as judged by formation of G418 resistant colonies. The promoters of the beta globin, zeta globin, and mouse immunoglobulin kappa light chain genes are much less efficient that the SV-40 promoter. In contrast, in non-erythroid cells the gamma promoter is only 1-5% as efficient as the SV-40 promoter in generating G418 resistant colonies. In none of the cells is activity of the gamma-neo construct increased by the viral enhancers (SV-40 or MaSV) or a putative tissue specific cellular enhancer.

4. In experiments described in individual project report "Identification of Cis and trans-Acting Factors that Regulate the Human Gamma Globin Gene", the region around 200 bp upstream from the transcriptional start site of the gamma globin gene has been identified as being of potential regulatory significance. Hybrid promoters containing this segment of the gamma globin gene flanking region and required segments of the beta promoter have been constructed and introduced into K562 cells. These cells express the endogenous gamma but not the beta globin gene. The initial transformants examined fail to express the beta coding sequences under the control of the hybrid promoter. If these results are substantiated, the -200 segment would appear to have a negative rather than a positive influence on promoter function.

Significance to Biomedical Research and to the Institute Program:

Patients with transfusion dependent beta-thalassemia or sickle cell disease could benefit immensely from augmented gamma globin chain synthesis and increased formation of HbF. The ability to chronically increase HbF synthesis might reduce or eliminate the need for transfusions in thalassemic patients allowing iron stores to be reduced by chelation or phlebotomy. Studies designed to determine the mechanism of hemoglobin switching during development and its regulation are directed toward the long term objective of obtaining permanent and physiological activation of the human fetal globin genes.

Proposed Course of the Project:

A systematic approach of creating mutants and hybrid promoters should inevitably succeed in identifying sequences involved in erythroid specific and developmentally specific expression of the gamma globin genes. In experiments complementary to those described in individual project report "Cis and trans-Acting Sequences that Regulate the Human Gamma Globin Gene" we have designed strategies to isolate genes that encode trans-acting molecules. The gamma globin gene promoter driven neo-transcriptional unit has been introduced into Chinese hamster ovary cells by co-transfection using dihydrofolate reductase minigene. Clones that have stably integrated the constructs but that do not express the G418 resistance marker will be tested for trans-activation by introduction of 1) viral oncogenes, 2)DNA from transformed cells, and 3) normal human DNA, followed by selection in G418. Clones obtained that express the neo-transcription unit after incorporation of human DNA, will be tested to determine whether activation has occurred by cis or trans-active mechanisms. Clones in which

trans-activation is defined will be characterized and an effort made to isolate the DNA sequences encoding the transactivating molecules.

Publications:

Anagnou, N.P., Moulton, A.D., Keller, G., Karlsson, S., Papayannopoulou, T., Stamatoyannopoulos, G., and Nienhuis, A.W.: Cis-acting Sequences that Affect the Expression of the Human Fetal Gamma-globin Genes. In "Experimental Approaches for the Study of Hemoglobin Switching". G. Stamatoyannopoulos and A.W. Nienhuis (eds.). A.R. Liss, New York, 1985.

Fordis, M., Anagnou, N.P., Dean, A., Nienhuis, A.W. and Schechters, A. A beta globin gene, inactive in the K562 leukemic cell, functions normally in a heterologous expression systems. Proc. Natl. Acad. Sci. USA 81:4485-4489, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02314 03 CHB

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Function of Proto-Oncogenes in Human Hematopoietic Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Principle Investigator: A. W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

Others: P.J. Browning, M.D., Guest Worker, CHB, NHLBI
H.F. Bunn, M.D., Guest Worker, CHB, NHBLI
J. Holt, M.D., Medical Staff Fellow, CHB, NHBLI
S. O'Brien, M.D., Chief, Genetics, LUC, FCRF, NCI
V. Gopal, Ph.D., Senior Staff Fellow, CHB, NHLBI
P. Turner, Medical Technologist, CHB, NHLBI
C. Sherr, M.D., Dept. Tumor Cell Biol., St. Jude Children's Hospital, Memphis, TN.

COOPERATING UNITS *(if any)*

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St. Judes Children's Hospital, Memphis, TN

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to characterize the function of the c-fms proto-oncogene in hematopoietic cells. Using very sensitive S1 nuclease mapping, we have identified c-fms mRNA molecules in most normal tissues including bone marrow. Expression of c-fms mRNA coincides with acquisition of other phenotypic features of the moncyte-macrophage lineage on stimulation of HL-60 cells, a promyelocytic line with a phorbal ester. C-fms mRNA was also found in peripheral blood monocytes. Recently other workers have identified the c-fms gene product as the receptor for the monocyte-colony stimulating factor (M-CSF). Widespread distribution of c-fms mRNA in tissues other than those involved in hematopoiesis, demonstrated in our studies, suggest that M-CSF and its receptor may have a wider role in cell proliferation and differentiation. We had previously shown that the c-fms gene is deleted from the 5q⁻ chromosome, present in the bone marrow cells of patients with refractory anemia. We have now obtained evidence suggesting a decrease in c-fms mRNA in the monocytes of such patients. Given the central role of the monocyte in hematopoiesis, this gene deletion may be of pathogenic significance. To further characterize the c-fms gene product, cDNA clones have been isolated from a library constructed with normal human liver mRNA. One encodes for the entire internal tyrosine kinase domain. Fragments of this clone will be used to construct hybrid v-fms/c-fms clones with the objective of creating a functional M-CSF receptor that lacks transforming capabilities. These studies are also designed to identify modifications in the v-fms gene that result in its transforming ability.

440

Project Description:

Objectives:

RNA tumor viruses owe their transforming potential to genes acquired by transduction from the genome of animal cells. The transforming genes of retroviruses are called v-oncogenes, whereas their cellular homologues from which they are acquired are refered to as proto-oncogenes. Many lines of evidence suggest that the proto-oncogenes encode for products that are relevant to cell differentiation and/or proliferation. Therefore, identification of cells in which specific proto-oncogenes are found, and analysis of their expression are likely to give us insights into both the role of the proto-oncogene products in normal and abnormal cellular differentiation, and proliferation. We have centered our investigation around the fms proto-oncogene (c-fms). Our objectives were to:

1. Determine the cell and tissues in which the c-fms gene is Expressed.

2. Isolate the c-fms coding sequences from a library of cDNA clones.

3. Characterize the functional domains of the fms protein including those sequences involved in transformation.

4. Identify and study those tumors associated with abnormal c-fms expression.

Methods:

1. RNA was prepared from chimpanzee tissues by lysis of the cells in Guanidium thlocyanate followed by either LiCl precipitation or Cesium Chloride buoyant density centrifugation. RNA from normal human liver, bone marrow, and placenta were prepared in a similar fashion.

2. S_1 nuclease analysis, using genomic DNA fragments from the human c-fms gene as probes, was used to assay for the presence of complementary mRNA in RNA from various tissues.

3. Northern blot analysis was used to determine the size of the c-fms transcript and quality of the mRNA preparations.

4. A human liver cDNA library was obtained from Melloy Laboratory. This library was screened with a nick-translated v-fms probe to identify cDNA clones.

5. Characterization of the c-fms cDNA clones was achieved by restriction enzyme mapping, DNA sequencing, and Southern blotting.

6. Populations of bone marrow mononuclear cells were purified by Ficoll-Hypaque centrifugation.

7. HL60 cells were cultured in RPMI medium containing 10% fetal calf serum. These cells were induced to differentiate to macrophages by addition of the phorbol ester, TPA.

Major Findings:

1. Hematopoietic cell fractionation revealed demonstrated c-fms expression primarily in cells of the mononuclear phagocytic lineage.

2. S_1 nuclease analysis of mRNA samples prepared from chimpanzee tissues revealed widespread c-fms expression in nearly every tissue studied.

3. HL60 cells, and BeWo cells were the only tumor cell lines shown to express the c-fms gene. The expression in HL60 cells occurred after induction with the phorbol ester, TPA, and was associated with an increase in c-fos expression and a decrease in c-myc expression. These changes in proto-oncogene expression in HL60 cells were associated with decreased proliferative capacity and emergence of adherent macrophages.

4. We have isolated several cDNA clones from the human liver cDNA library with strong homology to the v-fms probe. The two clones characterized thus far represent the 1/2 of the c-fms coding sequence that encodes for the internal tyrosine kinase domain. We are presently in the process of characterizing three other more recently isolated cDNA clones.

5. DNA sequencing of one of the c-fms clones has revealed 95% homology to v-fms coding sequence.

Significance to Biomedical Research and the Program of the Institute:

This project was designed to extend molecular biological techniques to the analysis of hematopoietic cell differentiation and function. Our data obtained thus far has given us some new insights into the role of the c-fms gene. Recently, other investigators have presented data identifying the c-fms gene product as the receptor for M-CSF (monocyte-colony stimulating factor). The ligand, M-CSF, is a specific growth factor that stimulates differentiation of bone marrow progenitor cells along the monocyte/macrophage lineage. Monocytes have been shown to function either directly or through interaction with T-lymphocytes, to influence hematopoiesis. A proliferation/maturation defect in monocytes due to a decrease in receptor number such as in $5q^-$ syndrome might contribute to the observed hematopoietic defectsobserved in the $5q^-$ syndrome. Characterization of the c-fms gene and identification of the mutations in v-fms that give it transforming properties may give insight into the potential of this gene to cause neoplasia.

Proposed Course of the Project:

Our immediate objective is to complete the cDNA cloning and characterization of the c-fms. C-fms expression has been shown in one tumor cell line call BeWo which is a chromoblastoma cell line.

Characterization of the c-fms transcripts and comparing it with the normal will provide information regarding the transforming domain of this protein. The characterization of the cDNA clones will allow us to construct hybrid genes involving v-fms/c-fms coding sequences. If in fact a functional protein is made by these hybrid genes we can identify the transforming segment of v-fms and investigate the growth properties of cells that have acquired an M-CSF receptor. We intend to extend our analysis of patients with the 5q syndrome to characterize monocyte-macrophage function.

Publications:

Nienhuis, A.W., Bunn, H.F., Turner, P.H., Gopal, V.T., O'Brien, S., Nash, W., and Sherr, C.: Expression of the Human c-fms Proto-Oncogene in Hematopoietic cells and its deletion in the $5q^-$ syndrome. Cell. In press, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02315 03 CHB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Lymphocytes and Lymphokines in Aplastic Anemia

PRINCIPAL INVESTIGATOR *(List other professional personnal below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Neal Young, M.D., Chief, Cell Biology Section, CHB, NHLBI
Others: Leonidas Platanias, M.D., Visiting Fellow, CHB, NHLBI
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Keiya Ozawa, M.D., Ph.D., Visiting Scientist, CHB, NHLBI
Julie Djeu, PH.D., Senior Investigator, DV, BB
Nicholas Zoumbos, M.D., Ph.D., Visiting Fellow, CHB, NHLBI

COOPERATING UNITS *(if any)* Pedro Gascon, M.D., Ph.D., Visiting Fellow, CHB, NHLBI

Division of Virology, BB

LAB/BRANCH

Clinical Hematology Branch

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Previous work has shown that patients with aplastic anemia have an elevated number of activated suppressor lymphocytes (cell surface phenotype Leu 2+, Tac+, HLA-DR+) that overproduce gamma-interferon (g-IFN), the lymphokine responsible for hematopoietic suppression in vitro, and possibly in vivo. Molecular characterization of T cell antigen receptor gene rearrangements has shown that the abnormal suppressor cell population is not clonal in origin. The inhibitory potential of interferons was further clarified by the use of pure recombinant interferons (r-IFNs). Though r-IFNs directly inhibited hematopoietic progenitor cells, the synergy between small amounts of gamma interferon and increasing amounts of alpha interferon required the presence of auxiliary cells. The antibody specificities in ATG and ALG were evaluated to better define these preparations. Both ATG and ALG were found to contain antibodies directed against several T cell antigens; however no significant differences were seen between active ATG and ALG lots and an ALG lot with less activity in vivo. Lymphocyte subsets and lymphokine production from aplastic anemia patients were studied pre- and post-ATG. Though there was not a significant change in total or suppressor lymphocytes after ATG, the number of lymphocytes bearing the interleukin 2 receptor fell to the normal range in all patients who responded to therapy; in contrast, 60% of nonresponders still had increased Tac antigen expression. In 3 patients studied, this decrease occurred on suppressor but not helper lymphocytes. Interferon levels in the peripheral blood and bone marrow sera decreased in 8/9 patients; the 1 patient without a decrease was a nonresponder. These results are consistent with the hypothesis that some cases of aplastic anemia are caused by an abnormal population of activated suppressor cells which are eliminated after ATG therapy.

444

Project Description:

Objectives:

Several lines of evidence suggest an immune mechanism for the suppression of hematopoiesis characteristic of aplastic anemia. Syngeneic bone marrow transplants occassionally need prior immunosuppressive therapy to succeed. Treatment with anti-thymocyte globulin (ATG) or anti-lymphocyte globulin, potent immunosuppressive agents, results in transfusion-free remissions in 40-50% of patients. We have previously demonstrated multiple immunologic abnormalities in patients with aplastic anemia including increased production of interleukin 2 (IL-2) and decreased production of interleukin 1 in vivo. Perhaps more important, these patients were shown to have elevated number of activated suppressor cells (as determined by the cell surface phenotype Leu 2+, Tac+, HLA-DR+) which overproduced gamma-interferon (g-IFN). Gamma interferon, which was elevated in peripheral blood and bone marrow sera from these patients, was found to be the factor responsible for hematopoietic suppression in vitro under a variety of conditions. By implication, g-IFN was felt to be responsible for the in vivo bone marrow suppression in some cases of aplastic anemia.

As part of our participation in a multicenter trial evaluating the efficacy of ATG, we were able to follow these immune parameters before and after therapy. Correlation of these abnormalties with treatment outcome was done in an attempt to discern important pathogenic mechanisms from epiphenomemon. In addition, an important goal was to try to predict prospectively which patients would benefit from ATG treatment.

Other investigators have recently shown that the gene coding the beta chain of the T cell antigen receptor is composed of multiple variable, diversity, and joining regions. This gene undergoes rearrangement during T cell maturation in the thymus in an analogous manner to the immunoglobulin gene. Thus it was determined if the abnormal suppressor lymphocyte population was uniclonal or polyclonal by studying rearrangements of beta chain DNA by Southern analysis using a cloned segment of beta chain constant region DNA as a probe.

We and others have shown that interferons (IFNs) can suppress hematopoiesis in vitro, but the IFN preparations used were often less than 10% IFN by weight and likely contaminated with other proteins which affect hematopoietic colony formation. However the recent availability of pure IFNs derived by recombinant DNA technology allowed us to study the hematosuppressive effects of IFN's more accurately. As the bone marrow of aplastic anemia patients have elevated levels of IFN which has biochemical and immunological properties of both gamma and alpha interferon, we studied the interactions of these two interferon types.

The mechanism of action of ATG and ALG is poorly understood. We have previously shown that these preparations have both stimulatory and inhibitory actions on several lymphocyte functions. We have compared

various properties of active ATG and ALG batches to a less active lot of ALG to evaluate which properties may be important for their clinical effectiness. The active and inactive lots were identical in their ability to induce lymphocyte blastogenesis, hematopoietin production, and to bind to platlets, lymphocytes, and granulocytes. However, there was a reduced ability of the inactive lot to cause complement-mediated lysis of human T lymphocytes. In an attempt to better define these preparations, we defined the antibody specificities in ATG and ALG.

Methods:

Cell surface markers were measured using commercially available monoclonal antibodies directly congugated to either flouroisocyanate (FITC) or phycoerythrin (PE) and peripheral blood mononuclear cells with a flourescent activated cell sorter. The presence of the IL-2 receptor was determined with a specific monoclonal antibody (supplied by Dr. Tom Waldmann) and FITC-congugated sheep anti-mouse IgG. IL-2 was obtained by stimulating mononuclear cells with PHA and testing the supernatant for the ability to support the growth of an IL-2 dependent cell line (CT-6). IFN was measured by its ability to protect several cell lines from virus infection using a viral plaque assay.

The method of Southern blotting was used to detect the T cell receptor beta chain gene in peripheral blood cells, using a radiolabeled fragment of the constant region gene as a probe. Clonal rearrangements were detected by a dimunition of germline bands and the presence of additional new bands.

Bone marrow colonies were grown in methylcellulose using standard techniques. These colonies allowed the enumeration of hematopoietic progenitor cells including the early erthyroid precursor (BFU-E), the late erythroid precursor (CFU-E), and the granulocyte-macrophage precursor (CFU-C). The effects of recombinant gamma interferon (r-g-IFN) and alpha interferon (r-a-IFN) on colony formation were studied using the r-IFNs alone and in combination.

The antibody specificites in ATG/ALG were determined by the ability of these preparations to block the ability of specific monoclonal antibodies to bind to T cell antigens as determined by immunoprecipitation and polyacryalamide gel electrophoresis of radioiodinated cell membrane lysates, flourescent activated cell sorting (FACS), and cell surface receptor binding studies.

Results:

Lymphocyte Subsets and Lymphokines Pre- and Post-ATG: There was no difference in the absolute number of total, helper, or suppressor lymphocytes after ATG treatment when compared to pretreatment values. However there was a significant reduction in the expression of activation markers such as the IL-2 receptor and HLA-DR on T lymphocytes. Whereas the number of lymphocytes bearing the IL-2 receptor fell to the normal range in all patients who responded to

therapy, 60% of nonresponders still had increased IL-2 receptor expression. In 3 patients studied, this decrease occurred on suppressor but not helper lymphocytes. IFN levels in the peripheral blood and bone marrow sera decreased in 8/9 patients; the 1 patient without a decrease was a nonresponder. IL-2 production by peripheral blood mononuclear cells returned to normal levels in 4/5 patient but was not related with response to treatment. These results are consistent with the hypothesis that some cases of aplastic anemia are caused by an abnormal population of activated suppressor lymphocytes which overproduce g-IFN and are eliminated after ATG therapy.

T Cell Antigen Receptor Gene Rearrangement: Polyclonal T cell activation results in numerous T cell antigen receptor gene rearrangements, none of which occurs frequently enough to be detected by Southern blotting. Thus the germline bands are diminished without the addition of new bands. In contrast, uniclonal rearrangements result in the presence of new bands in addition to the dimunition of the germline bands. Mixing experiments with the DNA of a T cell line (which has a single rearrangement) and granulocytes (germline configuration) revealed that a clonal popualtion comprising 5% of the total cells can be detected by this analysis. To date, the analysis of peripheral blood cell DNA from 15 patients with aplastic anemia has revealed that the elevated numbers of activated suppressor cells is not clonal in origin.

Hematopoietic Inhibition by Recombinant Interferons: Both pure r-g-IFN and r-a-IFN were able to inhibit hematopoietic colony colony formation in a dose dependent manner. This inhibition was highly dependent on the culture conditions; increasing the amount of fetal calf serum or colony stimulating activity diminished r-IFNs inhibitory actions. Whereas small amounts of r-g-IFN acted synergistically with increasing amounts of r-a-IFN, the addition of small amounts of r-a-IFN did not increase the inhibitory potential of r-g-IFN alone. Both r-IFNs directly inhibited colony formation; however, synergy could not be demonstrated when erythroid progenitor cells were cultured free of auxiliary cells such as lymphocytes and macrophages. Thus the ability of g-IFN to inhibit hematopoiesis appeared to be the result of both direct and indirect effects.

Results of multicenter trial of ATG: A large clinical trial of ATG in aplastic anemia and related bone marrow failure diseases, directed by this branch and involving over 160 patients enrolled at NIH and 14 other major medical centers, was completed in September. Preliminary analysis of data show that ATG is effective therapy in patients with acute severe aplastic anemia and probably superior to conventional therapy with androgens in patients with chronic or moderate severity aplastic anemia. Patients in group I (acute severe disease) were randomized to receive ATG in either 10 day (N=39) or 28 day (N=36) courses. The rate to transfusion independence at 3 months with 10 days of therapy was 33% and with 28 days 47%. Patients in age groupings from infants to the seventh decade had approximately similar responses, but men had a significantly higher likelihood of response to transfusion independence than did women (53% vs. 33%). The second group of patients consisted of individuals

with moderate or chronic disease, who were randomized to recieve first either ATG (10 days) or high doses of a parenteral androgen. The response rate in evaluable patients to ATG was 25% and to androgens 6%. The third group of patients consisted of individuals with a variety of bone marrow states, all treated with ATG; two patients with PNH responded to ATG, but myelodsyplasia, myelofibrosis, and cellular bone marrow refractory anemias were unresponsive.

Antibody Specificities in ATG/ALG: FACS analysis revealed that ATG and ALG have antibody specificities against multiple T cell antigens including the T cell receptor antigen (Leu 4+), a pan T cell antigen (Leu 1+), HLA-DR, and the antigens associated with helper (Leu 3+) and suppressor (Leu 2+) functions. The "inactive" ALG lot had similar specificities, though some at a decreased titer. Immunoprecipitation experiments with HLA-DR and Leu 2 revealed that ATG/ALG directed bound to these antigens, therefore, the blocking seen with FACS analysis was not simply due to steric hinderance. Though activity against the IL-2 receptor could not be demonstrated by FACS, it was seen using a more sensitive cell surface binding study using a tritiated anti-IL-2 receptor monoclonal antibody.

Significance to Biomedical Research and to the Program of the Institute:

The detailed study of aplastic anemia has given insights into the regulation of normal hematopoiesis and the disordered regulation in bone marrow failure states. The in vitro effects of lymphocytes and their soluble products such as gamma interferon have been correlated with their probable effects in vivo . The similarity between the immunological abnormalities in patients with aplastic anemia and chronic viral infections has led to a search for a viral cause of human aplastic anemia (see Individual Project: Viruses and Bone Marrow Failure).

A large clinical trial has confirmed the responsiveness of aplastic anemia to ATG in patients with acute and chronic aplastic anemia. The study of the antibody specificities in ATG/ALG and the changes in immunologic parameters before and after therapy may help determine the mechanism of action of these therapeutic regimens. This insight may permit the development of more effective, specific, and less toxic treatments for this highly fatal disease. The prospective identification of patients who would ultimately respond to ATG/ALG treatment would be very helpful in choosing treatment strategies, as this therapy is less toxic than bone marrow transplantation.

Proposed Course of the Project:

Immunologic parameters in patients with aplastic anemia will continue to be studied. Tests to predict the response to ATG will be sought.

The effect of auxiliary cells on the ability of IFNs to inhibit hematopoietic colony formation will be studied. Competition between IFN and other growth factors like interleukin-3 will be explored. The

effect of other inhibitory lymphokines such as tumor necrosis factor will be evaluated.

Evidence for clonality of T cells in other hematologic diseases such as chronic myelogenous leukemia and T-gamma lymphocytosis with cytopenias will be studied.

Other antibody specificities in ATG such as those directed against NK cells and monocytes will be evaluated. Further attempts to identify the active agent within ATG/ALG are still being persued.

Publications:

1. Zoumbos, N.C., Djeu, J.Y., and Young, N.S.: Interferon is the suppressor of hematopoiesis generated by stimulated lymphocytes in vitro. J. Immunol. 133: 769-774, 1984.

2. Young, N., Zoumbos, N., Gascon, P., Platanias, L., and Raefsky, E.: The immunologic pathophysiology of aplastic anemia: implications for a viral etiology. In Peschle, C. and Rizzoli, C. (Eds.): New Trends in Experimental Hematology. Oncogenes, Stem Cells, Bone Marrow Transplantation. Rome, Ares Serono Symposia, 1984, pp. 117-126.

3. Zoumbos, N.C., Gacson, P., Djeu, J.Y., and Young, N.S.: Interferon is the mediator of hematopoietic suppression in aplastic anemia in vitro and possibly in vivo. Proc. Natl. Acad. Sci. USA. 82: 188-192, 1985.

4. Zoumbos, N.C., Gascon, P., Djeu, J.Y., Trost, S.R., and Young, N.S.: Circulating activated suppressor T lymphocytes in aplastic anemia. N. Engl. J. Med. 312: 257-265, 1985.

5. Gascon, P., Zoumbos, N.C., Scala, G., Djeu, J.Y., Moore, J.G., and Young, N.C.: Lymphokine Abnormalities in aplastic anemia: Implications for the Mechanism of action of antithymocyte globulin. Blood. 65; 407:413, 1985.

6. Zoumbos, N.C., Raefsky, E.L., and Young, N.S.: Lymphokines and hematopoiesis. Prog. Hematol. In press, 1985.

7. Raefsky, E.L., Platanias, L.C., Zoumbos, N.C., Young, N.C.: Studies of interferon as a regulator of hematopoietic cell proliferation. J. Immunol. In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02318 02 CHB

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Enhancer and Promoter Specificity of Immunoglobulin Genes

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigator.) (Name, title, laboratory, and institute effiliation)*

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Others: Arthur W. Nienhuis, Branch Chief, CHB, NHLBI
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COOPERATING UNITS *(if eny)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use stenderd unreduced type. Do not exceed the space provided.)*

Tissue-specific enhancers seem to play a major role in controlling developmentally regulated gene expression. Expression of rearranged immunoglobulin (Ig) genes introduced into both lymphoid and non-lymphoid cells has led to the identification of tissue-specific transcriptional enhancer sequences in the major intron between the J and C region of the Ig gene. We have shown that Ig promoter also contribtues to tissue specific expression of mouse Ig Kappa gene. Tissue-specificity of Ig gene enhancers and promoters could be due to their interaction with some, as yet unidentified, trans-acting regulatory factors that may be present only in lymphoid cells. We have constructed special plasmid vectors containing the coding sequences of a dominant selectable marker gene that confers resistance to neomycin. These coding sequences are driven by an Ig gene promoter and the various vectors contain different Ig gene enhancers. By introducing these hybrid genes into non-lymphoid cells, we have created recipient cell clones in which the hybrid gene is stably integrated and non-functional. These cell lines can be used to directly isolate genes that code for putative trans-acting regulatory factors. Isolation of these regulatory genes will greatly enhance our ability to understand the regulation of tissue-specific genes at the molecular level.

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Project Description:

Objectives and Methods:

The main objective of this study is to investigate the mechanism(s) of tissue-specific gene regulation. Introduction of rearranged immunoglobulin gene clones (both heavy and light chain) into a variety of cell types has shown the presence of "enhancer" elements in the intron between the J and C region of the gene. They are necessary for the expression of immunoglobulin genes in lymphoid cells. These results suggested the presence of lymphoid cell specific factors that may interact with the enhancer region of immunoglobulin genes to promote their exclusive expression in immunoglobulin producing cells. To study the role of such specific enhancer recognizing factors in regulating tissue-specific gene expression, we devised a scheme-involving gene transfer and recombinant DNA methodologies to directly isolate genes that code for such putative regulatory factors. Once the regulatory gene becomes available, it could be used to answer specific questions relating to the organization and interaction between structural and regulatory genes at the molecular level. The knowledge gained through these studies may be applied to other systems such as the developmental regulation of globin gene expression.

Methods:

We constructed plasmid vectors designed to facilitate isolation of the gene coding for the putative regulatory factor gene using a gene transfer method. Included in the vector were: (1) the coding sequences for a dominant selectable marker; (2) a promoter that is stringently dependent upon an enhancer for its function; and (3) tissue specific enhancer elements from various immunoglobulin genes.

As the dominant selectable marker we chose the bacterial "Neo" gene. After testing promoters of different genes, the mouse k chain immunoglobulin gene promoter was chosen because it as totally inactive without an enhancer (we used the enhancer from Harvey sarcoma virus LTR region to test the enhancer dependency of promoters). Enhancer regions from mouse K light chain, u heavy chain and human k light chain immunoglobulin genes were chosen to insert into the test vector. These fragments were linked appropriately and the constructions were completed using standard cloning vectors. The coding sequences that confer neomycin resistance were replaced with those for the bacterial gene for chloramphenicol acetyl transferase (CAT) to create vectors that could be used to study function of the immunoglobulin promoter-enhancer combination using a transient gene expression assay.

The next step was to determine whether these test gene vectors demonstrated tissue specificity as reflected by lack of function in non-lymphoid cells but function in myeloma cells. If this condition was satisfied, then the strategy is to isolate clones of mouse L cells (a non-lymphoid cell line) having the Neo test gene vector stably integrated in a non-expressible manner followed by its activation by transfection with mouse or human myeloma DNA. It might then be possible to clone a gene encoding a enhancer activator using conventional methods.

Major Findings:

The cell-type specificity of the Ig K gene promoter was characterized using the coding sequences of two test genes, chloramphenicol acetyl transferase (CAT) for transient expression assays or the gene that confers neomycin (NEO) resistance for stable transformation assays. The HaMuSV enhancer activated the Ig K promoter in myeloma cells regardless of the enhancer's orientation or position within the vector. The level of CAT activity observed with the various vectors ranged from 20-105 percent of that seen with pRSV Cat, a standard vector in which CAT gene expression depends on the strong enhancer and promoter from the Rous Sarcoma virus genome. In contrast, no CAT activity was generated in 3T3 cells transfected with vectors containing the Ig K gene promoter and the HaMuSV enhancer. Similarly, the Ig K gene promoter with 625 bp of 5' flanking sequence was inactive in L cells when introduced in vectors containing the HaMuSV enhancer. Activity of the Ig K gene promoter was observed only with the shorter promoter having 225 bp of 5' flanking sequence and then only with the HaMuSV enhancer in tandem orientation.

The ability of the HaMuSV enhancer to activate the Ig K gene promoter was also studied in a stable transformation assay using vectors containing the Neo gene. These results corroborated the results of the transient expression assays. Both the Ig enhancer and promoter can be shown to function independently in lymphoid cells with a non-tissue specific promoter or enhancer, respectively, but neither the Ig promoter or enhancer will function consistently in non-lymphoid cells. Thus, the differential behavior of the Ig K gene promoter, when activated by a "neutral" enhancer, in three murine cell lines suggests that promoter sequences contribute to the tissue specific expression of this gene.

We have also isolated a number of clones of L cells carrying the Neo gene in test vectors. These cells do not express the neo gene since they will not grow in neomycin. Southern analysis showed that these clones contain the hybrid neo gene with immunoglobulin promoter and enhancer, stably integrated without rearrangement. Transfection of selected clones with human myeloma DNA gave rise to neomycin resistant clones. However, the neomycin resistant phenotype of the initial clones was due to cis-activation of the hybrid gene rather than trans-activation (tested by the ability of DNA from the activated clones to give rise to neomycin resistant clones upon transfection into mouse L-cells). Almost all of mouse L-cell clones containing the hybrid Neo gene can be activated by the adenovirus E1A gene, that has previously been shown to cause trans-activation of other cellular genes.

Significance to Biomedical Research and to Institute Program:

This project is designed to investigate the regulation of tissue-specific genes at the molecular level. In addition to its fundamental biological significance, understanding control of developmentally regulated gene

expression may have clinical relevance in regard to the pathogenesis of various diseases including tumorogenesis due to unscheduled expression of developmentally regulated cellular oncogenes.

Proposed Course of the Project:

Adenovirus E1A activated mouse L-cell clones containing the hybrid Neo test gene will be analyzed to map the neomycin mRNA start site by S_1 analysis. We have already cloned the Ig K promoter with 225 bp of 5'-flanking sequences and 321 bp of the 5'-end of the neomycin resistance gene into M13 to make the probe for these analysis. Also, the DNA from human and mouse myeloma activated clones will be analyzed by transfection assays into mouse L-cells and L-cell clones containing the inactive Neo test gene to identify transformants that arose due to trans-activation. Such clones will be used to isolate the sequences responsible for trans-activation by constructing a cDNA library using cDNA subtraction techniques. Alternatively, if we identify trans-activated clones using human myeloma DNA instead of mouse myeloma DNA, the DNA from Neomycin resistant primary clones will be used to isolate secondary transformants by repeat transfection. The DNA from these cells can then be used to construct a bacteriophage or cosmid library for screening with a human repetitive sequence "Alu" probe. We hope to isolate the gene responsible for activation of the neomycin resistance gene by interaction of its product with the immunoglobulin gene promoter or enhancer.

Publications:

T. V. Gopal, T. Shimada, A. W. Baur, and A.W. Nienhuis. Promoter contributes to tissue specific expression of the mouse immunoglobulin Kappa gene. Science, In Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02319 02 CHB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Viruses and Bone Marrow Failure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Eric Raefsky, M.D., Medical Staff Fellow, CHB, NHLBI
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Gary Kurtzman, M.D., Medical Staff Fellow, CHB, NHLBI
Neal Young, M.D., Chief, Cell Biology Section, CHB, NHLBI
Maria Harrison, Research Assistant, CHB, NHLBI
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COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As our previous studies suggested that some cases of human aplastic anemia may be secondary to persistant viral infections, the study of virus-induced bone marrow suppression may give insights into the etiology and pathogenesis of this human disease. Feline leukemia virus (FeLV), a slowly transforming retrovirus, is the most common cause of erythroid hypoplasia and aplastic anemia in domestic animals. In vitro experiments have shown that certains strains can selectively inhibit the proliferation of the more mature erythroid progenitor (CFU-E), while the proliferation of the more primitive erythroid progenitor (BFU-E) and the myeloid progenitor (CFU-C) is unaffected. This effect may be related to the higher cycling rate of CFU-E. Feline panleukopenia virus (FPV), a feline parvovirus which usually causes leukopenia in vivo, has been shown to inhibit the proliferation of both myeloid and erythroid progenitor cells in both short- and long-term bone marrow cultures. In addition, a better system for titering FPV has been devised. Previous work has elucidated the pathophysiology of a human parvovirus that is responsible for transient aplastic crises in children with chronic hemolytic anemias. In an attempt to better analyze specimens for the presence of this virus, "Western" blotting and DNA "dot" blotting methods have been devised. Using cloned parvovirus as a probe, attempts have been made to detect intergrated parvovirus DNA in the genome of patients with aplastic anemia. As the etiolgy of most cases of human aplastic anemia is unknown, bone marrow and peripheral blood cultures from these patients were started in an attempt to recover a pathogenic virus. As the culture supernatants from some patients have low levels of reverse transcriptase activity, an enzyme felt to be unique to retroviruses, this suggests that some cases of human aplastic anemia may also be associated with a retroviral infection.

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Project Description:

Objectives:

The etiology of human aplastic anemia is unknown in the majority of cases. However evidence suggests that this disease may be secondary to a persistent viral infection in at least a proportion of cases (see Individual Project: Lymphokines and Lymphocytes in Aplastic Anemia). Lymphocytes of patients with aplastic anemia have dysregulated production of lymphokines such as interferon and interleukin 2, similar to those infected with feline leukemia virus (FeLV) or human T cell leukemia virus (HTLV). As in some viral infections, a high percentage of these circulating lymphocytes are activated, as demonstrated by the presence of interleukin 2 receptors and HLA-DR molecules on their cell surface. In addition, viral infections are common causes of bone marrow suppression in animals and have been implicated in some cases of human aplastic anemia. Thus the study of viral-induced bone marrow failure may give valuable insights into the etiology and pathogenesis of aplastic anemia in humans.

Though FeLV is well known to cause lymphomas and leukemias in infected cats, it more commonly causes hematosuppressive diseases such as erythroid hypoplasia or frank aplastic anemia. While the molecular biology of this slowly transforming retrovirus is well studied, its pathophysiology is not well understood. In particular the parameters that affect its ability to infect bone marrow cells in vitro, and their relevance to in vivo infection, are unclear.

Feline panleukopenia virus (FPV), a parvovirus, is also associated with bone marrow supression in infected cats. Though infection with this virus is most often self-limiting, active infection is often accompanied by leukopenia, and to a lesser degree by anemia and thrombocytopenia. The mechanism of this effect has not been studied with in vitro systems and the in vivo parameters that modulate the natural history of this infection are also poorly understood.

A human parvovirus has been found to be responsible for transient aplastic crises in children with chronic hemolytic anemias. Previous work has demonstated that this virus selectively inhibits the proliferation of erythroid precursor cells via a direct cytotoxic effect. It is unknown if this virus is also related to some forms of chronic aplastic anemia. As this class of virus is known to intergrate into the DNA of its host, viral sequences in human DNA were sought using cloned parvovirus as a probe.

In other human diseases such as T-cell leukemia and aquired immune deficiency syndrome (AIDS), the retroviral etiolgy was not elucidated until infected cells were grown in culture since these viruses cannot replicate in non-dividing cells. To determine if some cases of human aplastic anemia are also associated with retroviral infection, the bone marrow and peripheral blood mononuclear cells were grown in short-term cultures and assayed for the presence of retroviral particles.

Methods

Methods were previously optimized to grow feline bone marrow cells in methylcellulose cultures in order to enumeration colonies which are the progeny of the early erythroid precursor (BFU-E), the late erythroid precursor (CFU-E), and the myeloid precursor (CFU-C). Standard tritiated-thymidine suicide experiments were modified for this system to determine the cell cycling rate for each of these progenitor cells. Feline bone marrow cells were also grown in long term culture with and without viral infection. Indirect immunoflourescence was done to document FPV infection and a modified plaque assay was established to titer FPV. This involved immobilizing susceptible feline kidney cells in methylcellulose after infection with serial dilutions of the virus, and enumerating viral foci with specific antibody and radioiodinated Staph protein A.

Two methods were used to assay for the presence of human parvovirus in serum samples. Viral antigens were detected by "Western" blotting which involves transfer of protein electrophoretically to nitrocellulose and detection using sera from patients known to have IgG antibodies to the parvovirus and radioiodinated Staph protein A. The presence of viral DNA was determined by "dot" blotting sera onto nitrocellulose and developing using a radiolabeled cloned parvovirus probe. In addition "Southern Blotting" was performed using DNA obtained from total bone marrow and peripheral blood cells and radiolabeled cloned parvovirus as a probe.

Bone marrow and peripheral blood mononuclear cells from patients with aplastic anemia were grown in liquid culture in the presence of growth factors and anti-gamma interferon sera in an attempt to induce retroviral growth. The cultures were split twice weekly, the culture supernatant was assayed for reverse transcriptase activity, and electron microscopy was performed on the cells to look for retroviral particles. The reverse transcriptase assay measures the incorporation of tritiated thymidine triposphate with an oligo·dT poly·rA template-primer in the presence of appropriate cations.

Major Findings:

Pathogenesis of FeLV and FPV-induced bone marrow suppression: In vitro studies were done with FeLV/C, the subtype which causes an erythroid hypoplasia in vivo. This subtype inhibitd the formation of CFU-E-derived colonies in a dose-dependent manner, but appeared not to inhibit formation of CFU-C or BFU-E- derived colony formation. In general, the degree of CFU-E inhibition correlated with the cycling rate (40-60%) of this progenitor cell. Conversely, the more primitive CFU-C and BFU-E progenitor cells had a much lower rate of cell cycling (0-15%).

In contrast, in vitro infection of feline bone marrow cells with FPV resulted in inhibition of both myeloid (CFU-C) and erythroid (BFU-E, CFU-C) colonies in a dose dedendent manner. Similar results were seen when feline bone marrow long term cultures where similarly infected.

The ability of this virus to infect bone marrow cells was also demonstrated by indirect immunoflourescence. However infectivity could not be demonstrated using a fibroblast cell line, showing that inhibition was not a non-specific finding common to all cycling cells.

Assays for human parvovirus detection: For parvovirus antigen to be detected in the sera of infected patients by protein transfer to nitrocellulose (i.e., "Western" blotting), the virus had to be concentrated and isolated from of other serum proteins by ultracentrifugation through a sucrose gradient. Initial attempts to develop the blots using a monoclonal antibody directed against a core protein proved unsuccessful. However successful blots were obtained using sera from patients previously exposed to the parvovirus and known to contain polyclonal anti-parvovirus IgG. These confirmed the presence of three proteins with molecular weights of 80,000, 68,000, and 48,000 associated with the viral core.

Detection of parvovirus in sera was also done by assaying for the presence of viral DNA with a "dot" blot method. With this method parvovirus could be detected in 0.001 ul. of sera, a sensitivity similar to the more commonly used radioimmunoassay. As other parvoviruses integrate their DNA genome into host chromosomes as part of their replicative process, the presence of viral DNA in bone marrow and peripheral blood cells of patients with sickle cell disease and aplastic anemia were analyzed. Using moderately stringent conditions, no sequences homologous to the cloned parvovirus were detected in 20 patients studied to date (half had previously parvoviral infection as documented by the presence of serum antibodies against the virus). The sensitivity of this assay is 1 viral copy in 5% of cells.

Search for retrovirus in human aplastic anemia: To date, 2/25 patients with aplastic anemia tested had slightly elevated levels of reverse transcriptase activity in the supernatant of bone marrow and peripheral blood cultures on multiple samples. In contrast, 0/11 normal volunteers had similar activity. This activity was transient but could be passed for several generations by co-culturing with normal bone marrow or peripheral mononuclear cells. Attempts to infect permanent cell lines such as HUT 78, MOLT, and K562 have been unsuccessful to date. Electron microscopy of the cultured cells from one of these patients revealed occassional extracellular particles 100 nm. in diameter which were consistent with retroviral particles. In both patients, similar activity was not seen in subsequent cultures started after anti-thymocyte treatment.

Significance to Biomedical Research and to the Program of the Institute:
The understanding of the pathogenesis of viral-induced apastic anemia may lead to significant insights into the etiology and pathophysiology of human aplastic anemia. The finding of an association between some cases of human aplastic anemia and a chronic retroviral infection would considerably advance our understanding of the cause of aplastic anemia and may permit more specific and less toxic treatment regimens to be devised. The availability of sensitive and rapid diagnostic tests of the human parvovirus should allow easier detection

and study of this virus both in the laboratory and in epidemiological studies.

Proposed Course of the Project:

The effect of increasing the cell cycling rate of erythroid progenitors on FeLV-induced anemia will be studied both in vivo and in vitro. More detailed analysis of FPV infection, including electron microscopy and in vivo studies, is planned.

Attempts will be made to infect the cloned parvovirus into a permissible cell line in order to easily grow the virus and to study its mechanism of infection. Preparations of viral proteins will be obtained by transfecting cells with appropriate subclones of the viral genome. This will allow their biochemical characterization and evaluation of the role of these proteins in the pathogenesis of parvoviral-induced anemia. The role of parvoviral DNA regulatory sequences will also be studied.

Further characterization of cultures from patients with aplastic anemia and reverse transcriptase activity will be done by analyzing multiple template specificities, sucrose gradient ultracentrifugation, and further co-culture studies. More detailed electron microscopy is also planned to verify the presence of retroviral particles.

Publications:

1. Young, N., Harrison, M., Moore, J., Mortimer, P., and Humphries, R.K.: Direct demonstration of the human parvovirus in erythroid progenitor cells infected in vitro. J. Clin. Invest. 74:2024-2032, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02320 02 CHB

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Pharmacological Manipulation of HbF Synthesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies were designed to determine whether hydroxyurea, an S phase ctyotoxic drug, would cause significant augmentation of fetal hemoglobin in patients with sickle cell anemia and beta thalassemia. Increses in HbF could be documented by sensitive immunological methods in approximately half of the patients with sickle cell anemia but no increase in HbF synthesis or red cell production was observed in two patients with thalassemia. Hence, our attention was redirected to testing several drugs singly, or in combination, in experimental animals in an effort to define a cldinically useful regimen. 5-Azacytidine, hydroxyurea, and cytosine arabinoside each cause an increase in HbF in baboons and Rhesus monkeys although 5-azacytidine is approximately two fold more active than the other two drugs. DMSO alone has no effect on HbF production but it appears to augment the increment seen following 5-azacytidine administration. Additional drugs will be tested during the next year in an effort to futher explore the clinical potential and mechanism of action of these agents. effect of an inducer of hemoglobin synthesis (dimethyl sulfoxide) that may augment the fetal hemoglobin response to 5-azacytidine.

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Project Description:

Objectives:

Patients with severe beta thalassemia or sickle cell anemia could significantly benefit if hemoglobin F production could be consistently augmented. In thalassemia patients, increased synthesis of gamma globin chains (found in HbF) compensates for the deficiency of beta globin chain production. The imbalance in globin synthesis, characteristic of this syndrome, is partially corrected by the increased gamma globin production. Reduction of intracellular HbF concentration by replacement with HbF reduces the polymerization potential of intracellular sickle hemoglobin and therefore reduces the sickling "propensity" of red cells from these individuals.

Our initial studies revealed that 5-azacytidine is capable of increasing HbF production in humans with these two disorders. Two mechanisms seemed likely to explain this effect. The first involved DNA hypomethylation induced by the 5-azacytidine. The second was that 5-azacytidine and S phase specific cytotoxic agent perturbed erythroid stem cell kinetics, secondarily altering the fetal globin biosynthetic program of the progeny of these cells. Based on the second hypothesis, other investigators proposed that other cytotoxic agents (like hydroxyurea or cytosine arabinoside [Ara-C]) would also increase HbF synthesis. Because hydroxyurea appears to have little carcinogenic potential, we initially chose to test its efficacy in sickle cell anemia patients, deferring further use of 5-azacytidine. Our initial studies showed that the drug was active, and that the mechanism of increased fetal hemoglobin synthesis appeared to differ from 5-azacytidine. The increases in HbF were modest and significant hematological changes were not documented.

Our current goal with animal studies is to define the role of a differentiating agent, DMSO, on the HbF response. Recently, Ginder and his colleagues at the University of Iowa have shown that an embryonic globin gene can be reactivated in anemic chickens if the chickens are sequentially treated with 5-azacytidine followed by an inducer of erythroid differentiation, sodium butyrate. Administration of azacytidine, ara-c, or sodium butyrate alone had no effect on embryonic globin gene expression. Only the combination of azacytidine and butyrate was capable of inducing this gene, suggesting that 2 "hits" were required for embryonic gene activation in adult erythroid cells.

Methods:

1. Hydroxyurea, azacytidine, ara-c, DMSO and combinations of the above have been administered to baboons and rhesus monkeys rendered anemic by administration of oral phenylhydrazine.

2. The hematologic responses to these drugs has been monitored by measuring the percentage of reticulocytes that contain hemoglobin F, the percentage of hemoglobin F^- containing red cells, and percentage of HbF in the

peripheral blood. Standard hematologic measurements (CBC, differential counts, platelet counts and reticulocyte counts) are also performed on a regular basis.

Major Findings:

1. Azacytidine (4 mg/kg IV daily times 5), hydroxyurea (100 mg/kg po daily times 5), ara-c (2 mg/kg IV daily times 5), all cause a striking increase in fetal hemoglobin biosynthesis in baboon and rhesus monkeys treated with these drugs. DMSO alone (1.5 gm/kg IV every other day for three doses) had essentially no effect on fetal Hb production in these animals. However, the combination of 5-azacytidine and DMSO administered concurrently may have an augmented effect on fetal hemoglobin production over that observed with 5-azacytidine alone, similar to what was noted in anemic chickens treated with sodium butyrate and 5-azactyidine. Further experiments will be required to confirm this difference between 5-azacytidine alone and 5-azacytidine plus DMSO. Additionally, DMSO will be added to hydroxyurea and ara-c to see whether a similar augmentation occurs.

2. There is a consistent difference between the baboons and rhesus monkeys with respect to hemoglobin F response. The rhesus has less basal fetal hemoglobin with phenylhydrazine treatment, and the fetal response to cytotoxic agents by rhesus is approximately 1/2 that of baboons.

3. Azacytidine is consistently the most active drug in these experimental animals. However, the kinetics of the F reticulocyte response appears to be similar for all three drugs. This suggests to us that all the drugs may share one mechanism for altering fetal hemoglobin production, i.e., they all have an inductive effect on erythroid progenitors with a fetal program. However, 5-azacytidine may have an additional modulating effect because of its ability to alter DNA methylation directly. The ability of DMSO to augment azacytidine's HbF response might be based on a mechanism similar to that of butyrate induction of embryonic globin synthesis in chickens.

Significance to Biomedical Research and to Institute Program:

Sickle cell anemia and beta thalassemia are severe diseases that cause serious morbidity and mortality. The developmentally silenced but structurally normal gamma globin genes, if activated, could compensate for

the genetic defects of the beta globin genes in patients with these conditions. Furthermore, these studies represent the first attempts to treat genetic diseases by manipulation of gene expression.

Proposed Course of the Project:

Additional drug courses as described above will be administered to the anemic rhesus monkeys and baboons. A careful analysis of all the accumulated data will then be performed using standard statistical methods. These data will be used to plan additional experiments.

Publications:

Nienhuis, A.W., Ley, T.J., Humphries, R.K., Young, N.S., and Dover, G.: Pharmacological manipulation of fetal hemoglobin synthesis in patients with severe beta thalassemia. Ann. of N.Y. Acad. Sci., 445:198-211, 1985.

Dover, G.J., Humphries, R.K., Young, N.S., Ley, T.J., Boyer, S., Charache, S., and Nienhuis, A.W.: Pharmacologic manipulation of fetal hemoglobin synthesis. In Proceedings of the Fourth International Conference on Hemoglobin Switching, Alan R. Liss, New York, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02321 01 CHB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Inhibition of Oncogene Expression with Antisense RNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jeffrey Holt, M.D., Staff Fellow, CHB, NIHLBI

Others: T. Venkat Gopal, Ph.D., Senior Staff Fellow, CHB, NHLBI

Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These studies are designed to analyze the effects of oncogene expression on cell growth and differentiation. The functions of the proto-oncogenes c-fos and c-myc will be studied by observing the phenotype of both benign (immortalized) and malignant cells when oncogene expression is inhibited. Recombinant DNA vectors designed to produce "antisense RNA" have been introduced into mouse fibroblasts (3T3) and human myelomonocytic leukemia cells (HL60). These vectors contain a steroid inducible (MMTV) promoter which should theoretically allow regulated production of the "antisense RNA". Preliminary findings indicate that 10 fold fewer stable transformants are obtained when 3T3 cells are transfected with "antisense RNA" vector DNA in the presence of steroid, than in its absence or with appropriate vector controls. Inducible "antisense RNA" clones (stable transformants) will be identified and analyzed in two well-studied models of oncogene expression: 1) the of serum-deprived 3T3 cells cells to growth factors; and 2) phorbol ester induced differentiation of HL60 cells. Comparison of the biological response of clones with selective inhibition of c-fos or c-myc expression with the wild-type cells and with appropriate controls may further elucidate the role of these proto-oncogenes in cellular growth and differentiation.

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Project Description

Objectives:

Immortalized or malignant cell lines respond to certain growth factors (or phorbol esters) with changes in oncogene expression and a biologic response. Serum-deprived mouse fibroblasts (3T3) that are exposed to serum factors respond with a 5-50 fold increase in C-fos mRNA within 15 minutes. Several hours later, C-myc expression increases and cell division occurs. Human promyelocytic leukemia cells (HL60) differentiate to monocytes on exposure to phorbol esters (TPA). Earlier work in this laboratory and others has documented an increase in c-fos expression and a decrease in c-myc expression at the time of differentiation. "Antisense RNA" technology is based on an empirical observation that RNA complementary to messenger RNA will inhibit the expression of the cellular mRNA, presumably by hybridization and translation arrest. The object of this project is to further characterize the significance of changes in oncogene expression, by attempting to overcome cellular oncogene regulation via "antisense RNA" vectors. Serum-deprived 3T3 cells with "antisense RNA vectors (and appropriate controls) will be exposed to growth factors in the presence of steroids . If the "antisense RNA" vectors can inhibit the changes in oncogene expression than it should be clear whether these changesare necessary for 3T3 cell division. Similarly, if the "antisense RNA" vector can inhibit the changes in HL60 oncogene expression (e.g. the increase in c-fos with differentiation) than differentiation into monocytes should be inhibited (if these oncogenes are necessary for differentiation). An understanding of the mechanism of differentiation of leukemic cells may be useful in attempts to differentiate these cells in patients.

Methods:

1. DNA vectors are constructed using standard recombinant DNA techniques and transferred into tissue culture cell lines with DEAE-dextran or electroporation. Cells treated with appropriate growth factors and/or steroids are extracted using the guanidinium hydrochloride technique to recover RNA and DNA. DNA samples are analyzed by Southern blotting and hybridization to radiolabelled probes. RNA samples are analyzed by nuclease protection assay (S1 nuclease) or Northern blotting to quantitate the amount of specific RNA present in the cell lines. RNA probes for the "antisense" vector, c-fos and c-myc have been developed in this laboratory.

2. 3T3 cell lines will be grown to confluence and then serum-deprived. Following the addition of growth factors (e.g. platelet derived growth factor) samples will be analyzed for RNA and DNA at appropriate time intervals. HL60 cell lines will be exposed to TPA and analyzed in a similar manner.

Major Findings:

Mouse fibroblast (3T3) and human promyelocytic leukemia (HL60) stable transformants that contain "antisense c-fos" or appropriate control plasmids have been identified and are being characterized. Transfection of 3T3 cells with "antisense c-fos" in the presence of steroid produced a 10 fold reduction in the number of viable colonies compared to appropriate controls, or "antisense c-fos" without steroid. Further characterization of these clones is necessary to confirm that this inhibition is the result of a anti-sense RNA transcript.

Significance to Biomedical Research and the Institue Program:

The observation that certain cellular genes can produce tumors when incorporated into the genome of retroviruses and introduced into animals has implications for both oncogenesis and cellular regulation. The ability to selectively inhibit specific oncogenes in models of differentiation may allow more complete understanding of the roles of these cellular proto-oncogenes in cellular growth and differentiation. In addition, these "antisense"stable transformants may serve as cellular mutants allowing analytical dissection of the differentiation pathway.

Proposed Course of the Project:

Stable transformants of 3T3 and HL60 cell lines will be obtained for both "antisense" c-fos and c-myc; and appropriate vector controls. Addition of growth factors or inducers, in the presence or absence of steroids will be performed to determine whether the "antisense RNA" inhibits the biological effect. If inhibition occurs, then the cellular process may be studied at various times with and without steroids , to further characterize the relationship between oncogene expression and cellular differentiation and growth.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02322 01 CHB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Defects in Beta Thalassemia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principel Investigator.) (Name, title, laboratory, and institute affiliation)*

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Others: Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI
Nicholas P. Anagnou, M.D., Ph.D., Visiting Scientist, CHB, NHLBI
George Stamatoyannopoulos, M.D., and Thalia Papayannopoulos, M.D.,
Division of Medical Genetics, University of Washington, Seattle, WA.
Janet Ash Tobian, Ph.D., Staff Fellow, HFB, NICHHD
Michael Zasloff, M.D., Branch Chief, HGB, NICHHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use stendard unreduced type. Do not exceed the space provided.)*

These studies are designed to define and precisely characterize various molecular lesions occuring in the beta-globin gene cluster in patients with beta thalassemia or with syndromes associated with increased HbF production in adult life such as delta-beta thalassemia, or hereditary persistence of fetal hemoglobin (HPFH). To investigate the mechanism by which premature termination codons cause a quantitative deficiency of beta globin mRNA , precursor and spliced mRNA molecules are generated in vitro and in vivo. The processing and nuclear to cytoplasmic transport of these RNA molecules are studied by microinjection into nuclei of Xenopus oocytes.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives:

The beta thalassemia syndromes provide an excellent natural model to study the mechanisms of defective gene expression, resulting from point mutations (within or flanking the beta globin gene) or from variable size deletions that remove important sequences from the beta globin gene cluster. Deletion mutants give rise to particular clinical syndromes that are characterized by an increased production of fetal hemoglobin (HbF) in adult life (delta-beta thalassemia and HPFH). The delta-beta thalassemia and HPFH mutations are clinically distinguished by the phenotype produced in heterozygous individuals. Heterozygotes for delta-beta thalassemia mutations have 8-15% HbF distributed in 30-50% of their red cells. In contrast, individuals heterozygous for HPFH mutations have 20-30% HbF that is uniformly distributed in all red cells. Individuals with these disorders exhibit a milder clinical course than those with typical beta thalassemia, due to the beneficial effect of HbF on red cell production and survival. Characterization of the various deletions that have different effects on expression of the fetal globin genes may provide insight into the mechanisms that regulate the normal perinatal switch from fetal to adult hemoglobin synthesis. A thorough understanding of these mechanisms may permit novel approaches to manipulation of fetal globin expression for treatment of beta thalasseia and sickle cell anemia.

Many B^0 thalassemia mutations that cause premature termination of translation also lead to a quantitative reduction in beta globin mRNA. Our earlier studies have shown that this quantitative reduction arises because of some alteration in nuclear processing or nuclear to cytoplasmic transport of the defective mRNA species. Ongoing studies are attempting to elucidate the relationship between the translatability of an mRNA and its intranuclear metabolism.

Methods:

1. A vector in which the SP6 bacteriophage promoter had been linked to the beta globin gene was obtained from Dr. Michael Green of Harvard University. Recombinant DNA methods are used to construct hybrid genes so that specific thalassemic mutations are introduced . SP6 polymerase (commercially available) is used to generate a precursor or spliced RNA transcript. The synthetic SP6 RNAs are injected into either the nucleus or cytoplasm of Xenopus oocytes and the oocytes are fractionated into nuclear and cytoplasmic compartments at appropriate time intervals.

2. The human zeta (embryonic alpha) globin gene is transcribed accurately and efficiently in Xenopus oocytes, but the human beta globin gene is not. Because of the apparent nuclear instability and slow transport of the synthetic SP6 beta globin RNAs, hybrid beta globin genes with a zeta globin promoter were constructed. DNA from these hybrids is injected into the Xenopus oocyte nucleus which transcribes the DNA; producing RNA *in vivo* . The B^0-39 thalassemia zeta-beta hybrid was

constructed using recombinant DNA techniques. A mutant beta globin gene with a 3 base pair deletion in exon II was constructed, to allow simultaneous analysis and comparison of processing and transport of the wild-type and B^0-39 thalassemic RNAs by S_1 nuclease assay.

Major findings:

1. The synthetic SP6 beta globin message (without introns) for both the wild-type and B^0-39 thalassemia genes were injected into the oocyte cytoplasm and showed equivalent stability over a 16 hour period. This finding is consistent with the prior observation (in this laboratory) that wild-type and B^0-39 thalassemia mRNAs exhibit similar cytoplasmic stability in Hela and Cos cells.

2. The synthetic SP6 beta globin messages (without introns) were injected into the nucleus and the cells were fractionated. Transport from nucleus to cytoplasm occurred slowly with only fifty percent of the message present in the cytoplasm at two hours. There was also nuclear degradation of both the wild-type and B^0-39 thalassemic message during the first hour after injection, and occasional leakage of RNA from nucleus to cytoplasm. These studies suggested that less B^0-39 message was transported to the cytoplasm, but were difficult to evaluate. The synthetic SP6 beta globin precursors(with introns) for both the wild-type and B^0-39 thalassemia genes showed extensive degradation in the nucleus and less than 10% of the transcripts were spliced at the intron I- exon II junction after 24 hours. No significant transport of spliced message was observed for either precursor gene.

Characterization of the intranuclear metabolism of the zeta-beta hybrid wild-type and B^0-39 thalassemia genes is in progress. The wild-type zeta-beta gene is accurately and efficiently transcribed from the canonical (zeta) cap site and a portion of the message is correctly terminated at the 3' end.

Significance to Biomedical Research and the Institute Program:

Homozygous beta thalssemia and the related disorders of increased HbF synthesis in the adult life are among the more common genetic diseases and may cause morbidity to affected individuals who are homozygous for these disorders. Molecular analysis and thorough characterization of these deletions and point mutations allows understanding of the genetic basis of these diseases and also will provide new insights into molecular mechanisms involved in the expression of genes in human cells.

Proposed Course of the Project:

Molecular characterization of new deletion mutants in either the beta or alpha globin cluster will be continued . The nuclear stability and nuclear to cytoplasmic transport of the wild-type and B^0-39 thalassemia zeta-beta hybrid genes will be compared. Any differences that are identified will be studied by introduction of a specific suppressor transfer RNA gene which suppresses the stop codon at the B^0-39 position.

Publications:

1. Anagnou, N.P., Papayannopoulou, T., Stamatoyannopoulos, G., and Nienhuis, A.W. Structurally Diverse Molecular Deletions in the Beta Globin Gene Cluster Exhibit an Identical Phenotype on Interaction with the B^S Gene. Blood 65:1245-1251, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02323 01 CHB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of the bone marrow defect in PNH

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jeffrey G. Moore, B.A., Chemist, CHB, NHLBI

Others:

Neal S. Young, M.D., Chief of Cell Biology Section, CHB, NHLBI
Micheal M. Frank, M.D., Clinical Director, LCI, NIAID

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia associated with a bone marrow defect in which erythrocytes develop an abnormal sensitivity to lysis by complement. The number of erythroid and myeloid progenitors, as detected by culture in a methylcellulose colony assay, were significantly decreased in bone marrow and blood of PNH patients. A higher proportion of the most primitive progenitors (BFU-E) were in cell cycle in PNH patients than normal individuals or other hemolytic anemias, which may be a result of increased recruitment due to destruction of maturing cells. To identify at what stage during differentiation cells became susceptible to lysis, progenitors and their in vitro progeny were incubated under conditions that promoted complement-mediated lysis. The progenitors were insensitive to lysis; the progeny were variably sensitive to lysis, indicating that the susceptibility of cells to lysis occurred late during erythropoiesis. In addition, we found that the proportion of abnormal cells within each erythroid colony did not fit the expected result of two separate populations of progenitors giving rise to either completely defective or normal progeny. To further test the hypothesis that one populaiton of progenitors exists in the PNH bone marrow, we used a polyclonal antiserum to a cell surface protein missing on defective cells. This functionally important molecule, called decay accelerating factor (DAF), regulates the deposition of the complement component C3b on the cell membrane. Using flow microfluorometry to measure DAF expression, two populations of red blood cells (rbc's) were detected in PNH patients, one corresponding to the negative control and the other to the distribution of DAF on normal rbc's. The negative population was reduced when cells were incubated with acidified serum prior to analysis. DAF expression was measured on erythroid progenitors and their in vitro normoblast progeny. In parallel with the previous studies, DAF was found present on the progenitors (BFU-E and CFU-E) and mostly absent on the in vitro normoblast progeny.

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Project Description:

Objectives:

PNH is a disease characterized by the abnormal sensitivity of erythrocytes to complement-mediated lysis. Typically patients are pancytopenic and have a bone marrow morphology that is normocellular or hypercellular. Many patients with PNH ultimately develop aplastic anemia. The insult responsible for this acquired stem cell defect is unknown. We used in vitro colony assays and flow microfluorometry to follow the generation of complement-sensitive cells during erythroid differentiation and to test the hypothesis that two populations of stem cells coexist in the marrow and to determine at what stage during development cells become susceptible to complement action.

Significant findings:

Properties of erythroid progenitors in the bone marrow and peripheral blood of PNH patients

The number of erythroid and myeloid progenitors detected using a methylcellulose colony assay was greatly reduced in PNH bone marrow and peripheral blood. Compared to normal individuals or other hemolytic anemia patients, the number of primitive erythroid progenitors (BFU-E) in cell cycle, measured by the ^{3}H-thymidine suicide assay, was markedly increased in PNH patients. These data suggested that chronic depletion of precursor cells and increased recruitment of progenitors may explain the development of bone marrow failure in some patients. To determine what stage of differentiation erythroid cells became susceptible to complement lysis, the progenitors and their in vitro normoblast progeny were incubated with acidified human serum to promote complement-mediated lysis. The progenitors were resistant to complement action; however the normoblast progeny of the BFU-E were variably sensitive to lysis, suggesting that erythroid cells in PNH become susceptible to lysis late in differentiation. In contrast to the expected result of BFU-E giving rise to colonies consisting of either completely normal or abnormal cells, we found that BFU-E also generated colonies that had both types of cells and that the proportion of defective cells increased as colony size increased. These data suggested that a single population of progenitors exists in the PNH bone marrow generating normal and abnormal rbc's.

A complement regulatory protein is present on PNH progenitors and lost during erythropoiesis in vitro

We followed the expression during erythropoiesis of a cell surface protein called decay accelerating factor (DAF), which regulates the deposition of the complement component C3b on the membrane. DAF expression was measured by the addition of a polyclonal antiserum and a FITC-conjugated second antibody to the cells and analyzed by flow

microfluorometry. PNH erythrocytes showed two populations of cells, one corresponding to the negative control and the other similar to DAF expression seen on normal rbc's. The negative population could be partially eliminated if the cells were exposed to complement lysis prior to analysis, indicating that DAF is functionally related to the disease in vitro. DAF was expressed in variable amounts on all cells in the circulation of normal persons. Other cells in PNH patients consistently showed decreased DAF expression, supporting the hypothesis that the PNH defect is present at the stem cell level. Bone marrow mononuclear cells from PNH patients and normal persons, which contained the hematopoietic progenitors, were sorted by flow microfluorometry on the basis of DAF expression. The DAF positive and DAF negative fractions were cultured separately and the resultant colonies enumerated. Colonies were detected solely in the DAF positive fraction, suggesting that all PNH progenitors express the DAF antigen. However, when the in vitro normoblast progeny from the patients was analyzed, mostly DAF negative cells were detected; normal erythroid progeny expressed DAF. These data further support the hypothesis that all PNH progenitors are genotypically defective and that late in differentiation there is a chance that cells will lose DAF from the cell surface and become susceptible to complement lysis.

Significance to biomedical research and to the program of the Institute:

A defective stem cell pool generates red blood cells that are abnormally sensitive to complement lysis in paroxysmal nocturnal hemoglobinuria (PNH). Understanding the biological mechanism of the membrane defect in PNH may allow for the development of specific therapeutic modalities to treat these patients. In addition, PNH provides an interesting model system to study hematopoietic differentiation and stem cell depletion leading to aplastic anemia.

Proposed course of the project:

We plan to determine the mechanism by which a complement regulatory protein (DAF) is lost during PNH erythropoiesis, rendering rbc's vulnerable to complement lysis. Two possibilities for dysregulation will be tested: 1) a developmental defect causing decreased synthesis of DAF; 2) DAF is synthesized normally but the molecule is not properly inserted in the cell membrane. The latter hypothesis is favored since many unrelated proteins are missing or abnormally expressed on PNH cell membranes. The biosynthesis of DAF will be studied using a radiolabeled amino acid to test these hypotheses.

Publications:

Moore, JG, MM Frank, HJ Muller-Eberhard, and NS Young. Decay accelerating factor is present on PNH progenitors and lost during erythropoiesis in vitro. J. Exp. Med. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02324 01 CHB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Identification of Cis and Transacting Elements that Regulate Human γ Globin Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Timothy J. Ley, M.D., Senior Investigator, CHB, NHLBI
Others: David Bodine, Ph.D., Guest Worker, CHB, NHLBI
Walter Gray, M.D., Staff Fellow, CHB, NHLBI
Lyn Mickley, Medical Technologist, CHB, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Understanding the normal regulation of the human γ globin genes is important for two major reasons: 1) This knowledge will provide new insight into the molecular mechanisms of gene activation and repression during developmental periods, and 2) knowledge of the normal function of the γ genes may allow us to devise new strategies to reactivate these genes in patients with disorders of the adult β globin gene. These studies are designed to analyze the molecular basis of human γ gene activation and repression, by defining the cis and transacting factors that regulate γ gene expression. Several laboratories have recently described point mutations approximately 200 nt upstream from the γ gene CAP site. These mutations are strongly associated with Hereditary Persistence of Fetal Hemoglobin. One mechanism by which the γ globin gene may be activated by these mutations could involve altered binding of regulatory proteins to this region. We have intensively studied the -200 region, and have found that an S1 nuclease hypersensitive site exists here in supercoiled plasmids, and that nuclear extracts from tissue culture cells contain proteins that bind to this region of DNA in vitro. A single nucleotide substitution at position -202 inactivates both protein binding and the S1 nuclease hypersensitive site. We have also performed a series of experiments designed to determine whether the γ gene promoter is under the influence of a tissue-specific enhancer. Initial results indicate that exogenous γ gene promoters are enhancer-dependent in a fetal erythroid cell line (K562), implying that the endogenous γ gene promoters may also be under the influence of an enhancer. An extensive survey of the γ globin gene region for an endogenous enhancer has revealed that a fragment 3′ to the human Aγ globin gene contains an enhancing element. The exact localization of this enhancing element, and its possible function in vivo, are currently under active investigation.

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Project Description:
Objectives:

The human γ globin genes become active during the 8th to 10th week of gestation, and remain so until shortly before birth, when they are repressed by unknown mechanisms. During the period when the γ globin genes are active, DNA near these genes is hypomethylated, and DNase I hypersensitive sites (indicative of active chromatin structure) are present near the promoters of these genes. These changes are probably epiphenomina of the activation process, and are probably necessary for γ globin gene expression. However, they do not explain what the primary influences of γ gene expression might be, nor do they point to specific sequences in or around these genes that might be responsible for their ordered expression.

Our studies are designed to define the cis and transacting regulatory influences of γ gene expression. Knowledge of these sequences will be important not only for understanding the general problem of developmental switching, but also for designing new strategies to permanently reactivate the γ globin genes in patients with Sickle Cell Anemia and β-thalassemia. These individuals suffer because of defects in adult β globin genes, which are activated shortly before birth. Previous experiments performed in this laboratory have demonstrated that it is possible to reactivate γ globin gene expression using a variety of cytotoxic agents. Increased γ gene expression can compensate for deficient β globin gene expression in patients with β-thalassemia, and can reduce the amount of sickle hemoglobin produced in patients with Sickle Cell Anemia. A safe and effective way of permanently reactivating the structurally normal (but functionally repressed) γ globin genes in these patients would probably provide a cure for these severe diseases. These studies represent a continuing search for clues regarding the normal regulation of the fetal hemoglobin genes.

Methods

1. S_1 nuclease hypersensitive sites in the γ globin gene promoter were mapped using techniques described by Efstratiadis and colleagues (Cell 35: 8137-8140, 1983). Basically, a DNA fragment containing the human γ gene promoter region (-302 to +33 of the $^{A}\gamma$ gene) was subcloned into puc 9. This plasmid was then opened at a unique restriction site, phosphatased, end-labelled with T4 kinase and $\gamma\ ^{32}P$ ATP, and ligated in the presence of ethidium bromide. Negatively supercoiled molecules are then isolated on non-denaturing gels, purified, subjected to S_1 nuclease digestion under limiting conditions, and then cut with various restriction endonucleases. The resulting fragments are electrophorsed on denaturing polyacrylamide gels.

2. Site directed mutagenesis in M13 was performed essentially as described by Zoller and Smith (DNA 3: 479, 1984). Basically, we designed all of the nucleotide primers with single nucleotide substitutions in the -200 region of the $^{A}\gamma$ globin gene promoter. These oligomers were synthesized by Pharmacia-P-L, and were annealed to the wild-type -200 region cloned in M13mp19. A gapped heteroduplex was formed, containing a single nucleotide mismatch as defined by the primer. These heteroduplexes were transfected into E. coli JM101, and mutated clones were subsequently identified based on their

melting profiles with wild type probes. Three mutants have thus far been created: 1) -202 C to G, a naturally occurring mutant described by Collins et al., in 1984. 2) -196 C to G, a naturally occurring mutant described by Otto-Lenghi in 1983, and -199 T to A, an artificially mutant selected at random. The first of these mutants has been confirmed (by restriction endonuclease analysis) to contain the C to G substitution, and the second two mutants are currently being subcloned into puc 9 for subsequent sequence and structural analysis.

3. Studies of DNA-protein interactions have been performed using a variety of techniques. An Exo III assay has been used to define DNA protein interactions at single nucleotide resolution. Basically, very small fragments of DNA (less than 350 base pairs) are uniquely end-labelled, allowed to interact with various protein extracts, and digested with the 3' - 5' processive enzyme Exonuclease III. This enzyme destroys DNA molecules in a step-wise fashion from the 3' towards the 5' end. Since the label is placed at the 5' end, the enzyme essentially works toward the label. If the enzymes encounters any tightly bound proteins on the DNA, it is stopped and a new fragment is created. DNA filter-binding assays have been performed essentially as described by Emerson and Felsenfeld (PNAS 8: 95, 1984). Southwestern blotting is performed as essentially as described by Bowen, et al., (Nucleic Acids Res. 8: 1-20, 1980). Basically, nuclear extracts from a variety of cell types are electrophoresed on 13% SDS-Page gels (non-reducing), transferred to nitrocellulose filters electrophoretically, and hybridized to end-labelled double-stranded DNA probes under non-denaturing conditions.

4. A γ gene promoter fragment extending from -302 to +33 nucleotides was fused to the cloned chloramphenicol acetyltransferase (CAT) gene in a Puc 9 vector. A movable γ-CAT cartridge was then created with Sal-I ends. Subsequently, a large number of restriction endonuclease fragments from the β globin gene cluster were subcloned into Puc 9. Then, the γ-CAT cartridge was inserted into the Sal I site of this series of plasmids. These plasmids were transfected into a variety of cell lines by calcium-phosphate mediated DNA transfer, and the cells were then allowed to grow in culture for 2-3 days. At that time, the cells were lysed by sonication, and total cellular proteins were prepared. The protein concentrations were adjusted and finally, the ability of the cellular extracts to acetylate C14 labelled chloramphenicol was analyzed. Since no chloramphenicol acetyltransferase is produced by eukaryotic cells, the background signal is nil. A higher signal level implies increased function of the γ promoter which drives the CAT gene. The CAT assay was performed essentially as described by Gorman and colleaques (PNAS 79: 677-681, 1982).

Major Findings:

1. The γ globin gene promoter region contains an S_1 nuclease hypersensitive site located approximately 200 nucleotides upstream from the CAP site of this gene in supercoiled plasmids. Other investigators have similarly noted an S_1 nuclease hypersensitive site approximately 200 nucleotides upstream from the β^A globin gene of chickens and for the human β globin gene. These S_1 hypersensitive sites correspond to S_1 nuclease sensitive regions found in chromatin from cells expressing these genes, implying that they are of

functional importance. Substitution of a guanosine residue for the normal cytosine residue at position -202 completely abolishes S_1 nuclease hypersensitivity in this region. Since the same mutation upstream from the $^{G}\gamma$ gene is tightly linked to an HPFH phenotype in Jamaicans, we believe that this mutation probably causes activation of the $^{G}\gamma$ gene by altering the secondary structure of DNA found in this region in vivo.

2. Nuclear extracts from K562 cells contain an activity that inhibits exonuclease III in the -200 region of a linear duplex containing the $^{A}\gamma$ gene promoter. This activity behaves as a protein, since it is heat sensitive and can also be destoryed by incubation with trypsin. Again, introduction of the -202 C to G mutation abolishes the Exo III inhibition in this region, implying that the binding of a protein to this region is somehow altered by the mutation.

3. Nuclear extracts from K562 cells contain a variety of DNA binding proteins as defined by filter binding and the Southwestern blot assay. The nature of these binding proteins is not yet been deduced, but the pattern from K562 cells, HL60 cells and HeLa cells is clearly very different. The γ gene promoter probe essentially identifies the same proteins in uninduced and induced K562 cell nuclei. Furthermore, the wild type and -202 mutated γ promoter probes identify the same series of proteins in this assay. Therefore, our initial studies have not identified a unique protein with altered binding to the -200 region.

4. The γ globin promoter-CAT gene construction is expressed at high levels in non-erythroid cells (L cells, HeLa cells, etc). However, this γ-CAT gene is expressed only at very low levels in the embryonic-fetal erythroid cell line, K562. As anticipated, expression of the γ-CAT gene was markedly increased when the vectors introduced contained known enhancing elements from viral sources. The SV40 enhancer element increased γ-CAT expression by 200 fold over the enhancerless plasmids, while murine and avian virus enhancers caused a 20-fold augmentation of γ-CAT expression. Therefore, an exogenous γ gene promoter is under the influence of an enhancing element in K562 cells. This may mean that the endogenous γ promoter is similarly under the influence of an enhancer.

For this reason, we subcloned 26 restriction endonuclease fragments into a γ-CAT containing vector and examined γ-CAT expression. Thus far, only one fragment obtained from the human γ globin gene region has cause increased expression of the γ-CAT gene. This 2.3 kb Eco RI fragment is located 1.5 kb 3' to the human $^{A}\gamma$ gene, and consistently causes a 20 fold increase in γ CAT expression. DNA sequence analysis of this fragment revealed an 11 base pair segment that was highly homologous (10 of 11 nucleotides) to the SV40 enhancer core element. No other enhancer core-like elements were found in this fragment. However, we could not detect proteins binding to this region in an exonuclease III assay, nor could we detect an S_1 hypersensitive site in a small fragment of DNA containing this region. The 2.3 kb region has been subdivided into a variety of smaller fragments to precisely localize the enhancing activity. These experiments are currently in progress.

5. We have examined the structure of the 2.3 kb Eco RI fragment noted above in DNA obtained from tissues where the γ gene is either expressed or not expressed. Other investigators have shown that a methylation sensitive site near the 3' end of this fragment is consistently hypomethylated in tissues which express either the fetal or adult globin genes. Methylation patterns of 2 other methylation sensitive sites in this fragment do not correlate with γ or β gene expression. The chromatin structure of this region of DNA has also been examined. No DNase I hypersensitive sites were found in the 2.3 kb Eco fragment in chromatin derived from HL60 cells, K562 cells, or adult human erythroid cells obtained from a patient with Sickle Cell Anemia.

Significance to Biomedical Research and to Institute Program:

Identification of the proteins that regulate the γ globin gene and the DNA sequences with which they interact will provide new insights into mechanisms of gene control. The study of naturally occurring mutations that cause activation of the γ genes has provided new insights about one possible regulatory region. Manipulation of the influences that regulate γ gene expression may help us create new strategies for permanent reactivation of the γ genes in the patients with Sickle Cell Anemia and β-thalassemia.

Proposed Course of the Project:

We will continue to study point mutations in the -200 region of the γ gene promoter, and their effect on DNA structure and protein binding in this region. An effort to identify and characterize the protein(s) that bind in this region is currently underway.

We are currently subdividing the 2.3 kb Eco RI fragment into subfragments to localize the enhancing activity. Further studies of the normal structure and function of this region in erythroid and non-erythroid cells are in progress. Furthermore, the ability of this fragment to augment expression of enhancer-dependent genes in heterologous cells is underway. These studies should provide information that will help to define the role of this sequence in normal erythroid cells.

Publications: none

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02325 01 CHB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Treatment of Chronic Myelogenous Leukemia with Recombinant Interferon-Gamma

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Gary J. Kurtzman, M.D., Medical Staff Fellow, CHB, NHLBI
Others: Neal S. Young, M.D., Section Chief, CHB, NHLBI
Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI
Keiya Ozawa, M.D., Ph.D., Visiting Scientist, CHB, NHLBI
Leonidas Platanias, M.D., Visiting Scientist, CHB, NHLBI
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COOPERATING UNITS *(if any)*

Genentech, Inc., South San Francisco, CA
Biological Response Modifiers Program, FCRF, NCI, Frederick, MD
Division of Cancer Treatment, NCI

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unraduced type. Do not exceed the space provided.)*

Chronic myelogenous leukemia (CML) is characterized by increased formation of granulocytes and other blood forming elements which, with a median time of three years, is almost uniformly fatal. Conventional chemotherapeutic regimens for its initial chronic phase have failed to prevent transformation to acute leukemia or to significantly affect survival. Although bone marrow transplantation is effective therapy in a minority of patients, more effective treatment regimens for the chronic phase have to be designed. We previously demonstrated that gamma interferon (IFN-gamma) has potent suppressive effects on hematopoiesis in vitro and provided evidence implicating it in the pathogenesis of the hematopoietic suppression observed in aplastic anemia. Preliminary work has also demonstrated a similiar suppressive effect of IFN-gamma on the in vitro hematopoietic colony formation in patients with CML. This evidence, coupled with the in vivo suppression of myelopoiesis observed in patients treated with recombinant IFN-gamma for other disorders, makes this agent particularly well suited to the treatment of CML; preliminary studies support this. We have designed and received approval for a study of the treatment of patients with both the chronic phase and the accelerated phase (a transition phase from the chronic to the acute stages of the disease) with recombinant IFN-gamma. Patients in the chronic phase will be treated with continuous subcutaneous administration, for preliminary evidence would suggest that in order to be effective, maximum exposure time to IFN-gamma is needed. Patients in the accelerated phase will be treated with daily intramuscular administration. Several studies have already begun to study the biology of this unique disorder.

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Project Description:

Objectives:The objectives of this study are to evaluate the subcutaneous route as a means for the delivery of recombinant IFN-gamma as well as the efficacy of continuous exposure to this agent in inducing response in patients with the chronic phase of CML. If response is achieved, the ability of recombinant IFN-gamma to maintain the response will also be investigated. In addition, recombinant IFN-gamma administered by the intramuscular route will be investigated for the same properties in patients with the accelerated phase of the disease. Information regarding the pharmacology and toxicity of recombinant IFN-gamma will also be ascertained. We have been and will continue to investigate the in vitro colony characteristics of patient's hemato-poietic progenitors and the effect of recombinant IFN-gamma in this system. We are also studying cell surface phenotypes on immune effector cells in CML.

Approximately 90% of patients with CML have a characteristic chromosomal defect known as the Philadelphia chromosome. This arises from the translocation of a portion of the long arm of chromosome 9 to chromosome 22. The proto-oncogene, c-abl which resides on the long arm of chromosome 9 is transferred to chromosome 22 as part of the translocation. A modified oncogene-encoded protein, with segments contributed from both chromosomes is produced and may be involved in the pathogenesis of the malignant process. DNA probes have been developed, and we are currently using these to further delineate the gene rearrangement and abnormal gene expression in patients with CML.

Methods:

Recombinant IFN-gamma, produced by recombinant DNA technology, is kindly provided by Genentech, Inc., and has received approval by the FDA under investigator IND#2212 for the treatment of patients with CML. Patients in the chronic phase of the disease will receive from 0.01 - 0.5 mg/m2/day by continuous subcutaneous infusion using a Autosyringe Model AS3B or equivalent pump. Patients with the accelerated phase will initially receive 0.25mg/m2/day and if tolerated will be escalated to 0.5mg/m2/day. Patients will be monitored at intervals for response and toxicity.

Peripheral blood and bone marrow colonies are measured in methylcellulose using standard techniques and enumerated by their characteristic morphology. Interferon-gamma, kindly provided by Genentech, Inc. is included in these cultures in order to determine its effect.

Cell surface markers on peripheral blood and bone marrow cells are measured in a fluorescence-activated cell sorter using commercially available monoclonal antibodies. T cells obtained by rosetting with sheep red blood cells and adherent separated monocytes will also be studied.

The presence of interferon in serum or culture is measured in an antiviral assay by its ability to inhibit the infection of WISH cells, a human amniotic cell line, by vesicular stomatitis virus. A radio-immunoassay is commerically available (Centocor, Malvern, PA) that specifically measures IFN-gamma.

High molecular weight DNA from patients with CML as well as normal individuals is extracted from peripheral blood white blood cells or bone marrow nucleated cells and subjected to digestion by restriction endonucleases. These are electrophoresed on agarose gels, transferred to nylon membranes using the Southern blotting technique, and hybridized with P-32 labeled specific probes. These include probes for a specific region on chromosome 22, termed the breakpoint cluster region (bcr), involved in the translocation process and probes for the c-abl proto-oncogene normally located on chromosome 9. RNA is isolated using the guanidine hydrochloride technique and enriched for RNA containg poly A segments (i.e. messenger RNA). These RNA's are electrophoresed on agarose gels, transferred to nylon membranes using the Northern blotting technique, and hybridized to probes similiar to those described above.

Results:

Preliminary studies revealed that the number of hematopoietic progenitors that can be enumerated in the bone marrow of patients with CML is more variable than in normal individuals. In general, patients with CML have at least 5-10 times the number of progenitors per total mononuclear cell population in the bone marrow than normals. The typical colony in CML, be it myeloid (CFU-C) or erythroid (BFU-E), is larger (i.e. contains more cells) than those from normal donors. Recombinant IFN-gamma shows some suppressive effect on colony formation, however, the variability and lability of the interferon preparations used to date and the heterogeneity of the disorder mandates further study in a larger number of patients. In at least one patient with chronic phase CML interferon was present in normal levels in the bone marrow, and upon stimulation with phytohemaglutanin, peripheral blood mononuclear cells produced normal amounts of interferon. This conflicts with what can be inferred from the literature, and given the recent availability of recombinant IFN-gamma and more accurate techniques of detection, more patients will have to be studied.

Peripheral blood and bone marrow DNA and RNA has been prepared from both patients with CML and from normals. Work is currently under way using the molecular probes described above to further delineate the specific defects in these patients. Interestingly, of the four patients from whom we obtained samples, one patient lacks the characteristic Philadelphia chomosome (so-called Philadelphia negative CML) and rather has another cytogenetic defect, an additional chromosome 8. As the clinical course of Philadelphia chromosome negative CML is different

from the more common form of the disease (these patiets tend to have shorter survivals), work is in progress to determine if this patient has defects at the molecular level similiar to those in Philadelphia chromosome positive disease and if not, to more closely study this particular patient.

Significance to Biomedical Research and to the Program of the Institute:

CML is a common form of leukemia in adults, generally affecting persons in middle life. Clearly, if therapy is to prolong survival, it has to be administered during the chronic phase, before the disease evolves. A body of evidence already exists to document IFN-gamma's suppressive effects on hematopoieis, and preliminary data suggest an efficacy in the treatment of CML. The optimal route of delivery of IFN-gamma has yet to be developed given the apparent need for continual exposure to the agent, thereforethis study is also designed to evaluate the value of continuous subcutaneous administration. Intramuscular administration is effective in the small number of patients in the accelerated phase of the disease who have been studied, but more patients need to be evaluated. CML is also the first example of a human cancer where a chromosomal translocation leads to the formation of a modified oncogene-encoded protein which may be involved in the pathogenesis of the disease; work remains to further characterize the molecular biology of this disease.

The Clinical Hematology Branch has, for a number of years, devoted a large effort to the study of both ordered and disordered hematopoiesis. Significant contributions have been made to the understanding of aplastic anemia, a disease of inadequate hematopoiesis Chronic myelogenous leukemia is a disease arising at the level of a pluripotent stem cell giving rise to most, if not all of the blood cells. Continued study of CML will allow us to focus on a disorder of overproduction of the blood elements and allow us continue our investigation into the mechanisms of hematopoiesis from a different perspective. The study of blood and bone marrow specimens from patients with CML will be of value in the study of the role of oncogenes and oncogene products in both normal and disordered hematopoiesis. The mechanisms which allow for expression of this abnormal oncogene product apparently exclusively in CML may well provide insight into the regulation of hematopoiesis

Proposed Course of the Project:

The plan is to treat twenty patients with the chronic phase and fourteen to thirty patients in the accelerated phase in order to have a sufficient enough sample size in order to make statistically significant conclusions. If successful, it is anticipated that treatment will continue either in the same form or modified in some way as to maintain the responses achieved.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02326 01 CHB

PERIOD COVERED
October 1, 1984 - September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cloning and Characterization of a Sequence of Human DNA with homology to Adenovirus 5 and 12

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principel Investigator.) (Name, title, laboratory, and institute affilietion)*

Neal D. Epstein, M.D., Medical Staff Fellow, CHB, NHLBI
Others: Stefan Karlsson, M.D., PhD., Visiting Associate, CHB, NHLBI
Arthur Nienhuis, M.D., Branch Chief, CHB, NHLBI
Steve O'Brien, PhD., Branch Chief, LVC, Frederick, MD.

COOPERATING UNITS *(if any)*

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

During the course of gene transfer experiements into K562 cells using a modified adenovirus vector, it was noted that control K562 cell DNA showed a positive signal when probed with the entire adenovirus type 5 genome. Subsequently we have determined that this signal is also present in all human genomic DNA tested. An Sst I digest of human genomic DNA probed with the entire adenovirus 5 disclosed 3 bands that were shown to be homologous to distinct portions of the adenovirus genome. A sequence was isolated from a human cosmid library that corresponded to a particularly strong signal at 2.5 KB on a genomic human Sst I digest, probed with the first 9 Kb of the 5' end of adenovirus 2. This isolated clone has been shown to contain a 2.5 Kb Sst I frament homologous to a portion of the E1B coding region of adenovirus 2 and 5. It also has its own interesting structure in that this unit is repeated in tandem fashion approximately ten times in the cosmid clone. We have tentatively mapped this sequence, through the use of somatic cells, to human chromosome 4. The copy number of this sequence has been estimated as greater than 300. At present in situ hybridization is being performed and we are beginning to sequence the 2.5 Kb Sst I fragment.

PHS 6040 (Rev. 1/84) GPO 914-918

Objective:

At the outset of this project the finding of a positive signal with an adenoviral probe on a Southern blot of K562 genomic DNA suggested that transformation of the K562 cell line had originally involved an adenoviral infection. This was soon shown to be unlikely as Southern blots of normal human genomic DNA displayed the same signal. Subsequently our objective has been to further characterize those sequences in the human genome that are homologous to sequences in adenovirus. One such sequence was isolated and shown to be homologous to a portion of the E1B coding region of the adenovirus. The isolated sequence also had its own interesting structure in that it existed as a tandem repeat with very high copy number. Further objectives of this project include sequencing the repeat unit and subsequent examination of its structure for open reading frames and/or possible cis acting regions. RNA from different cell lines will be probed with the isolated DNA fragments for evidence of expression.

Methods Employed:

To date much of the work on this project has invovled Southern blots of human DNA with adenoviral probes. Standard techniques of cosmid cloning were used to isolate the 2.5 Kb tandem repeat unit. The mapping of the repeat unit has been done with DNA from rodent-human hybrids and is presently being pursued by the technique of in situ hybridization. We are now in the process of preparing truncated digests of the 2.5 Kb Sst I cloned fragment inserted into M13 to facilitate dideoxy M13 sequencing. RNA samples of Hel, HL-60, HeLa, K562, MEL and 293 cell lines are being analysed by RNA dot blot to be followed by Northern Blot and S_1 analysis if appropriate. Southern blots of DNA from different primates and mammals are also being done to examine the evolutionary pattern of this tandemly repeated sequence.

Major Findings:

The major findings of this project to date have been as follows:

1. Normal human genomic DNA contains several sequences homologous to regions of adenovirus type 2 and 5.

2. We have cloned one such sequence from a human cosmid library and have tentatively mapped it to chromosome 4.

3. This sequence is homologous to a portion of the E1B coding sequence of adenovirus 2 and 5.

4. This sequence has its own interesting structure in that a Southern blot of an Sst I digest of the cosmid clone discloses a 2.5 KB band of approximately 10 copies indicating a tandem repeat structure. Copy number as estimated by the strength of signal on a Southern Blot of

human genomic DNA probed with the 2.5 Kb piece is between 300 and 500 copies. This represents approximately between .01% to .02% of the DNA in chromosome 4.

5. Southern blots of somatic cell hybrid DNA shows a family of Sst I bands with homology to the cloned fragment. However, they are on different chromosomes and of much lower copy number.

Significance to Biomedical Research:

The finding of sequences in human DNA with homology to Adenovirus may be neuristic in several ways.

1. It may help in our understanding the relationship between man and human trophic viruses, i.e., viral origins as well as other possible interactions between viruses and man.

2. The cloning of sequences of human DNA with homology to a viral genome may be one way to isolate sequences of human DNA with important functions in the process of gene regulation whether these be cis or trans acting.

3. The isolated sequence may represent a new family of repetitive DNA.

4. The isolated sequence when further tested may be instrumental in the amplification of plasmids and thus able to increase the yield of useful molecules produced through recombinant DNA techniques.

Proposed Course:

As stated previously we are just now beginning to sequence the 2.5 Kb tandem repeat unit. Once this is accomplished a computer search for homology to the adenoviral genome and for open reading frames will be done. This may lead to testing of portions of this sequence for cis acting activity. If open reading frames exist, Northern blots and S_1 analysis will be used to identify transcripts. Southern blots of primate and other mammelian DNA will be probed with the 2.5 Kb sequence to determine the usefulness of this probe for evolutionary studies. Other adenoviral related sequences may be cloned from a human genomic library and subsequently characterized as described above.

Publications:

None

ANNUAL REPORT OF THE
LABORATORY OF EXPERIMENTAL ATHEROSCLEROSIS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1984 through September 30, 1985

Atherosclerosis is the underlying basis of most coronary artery disease, the leading cause of death in the United States. Because pathological cholesterol accumulation is central to the development of the atherosclerotic lesion, we have chosen to study this aspect of atherosclerosis.

Work in the Laboratory of Experimental Atherosclerosis is continuing in the examination of mechanisms of cellular cholesteryl ester accumulation that may be relevant to accumulation of cholesterol within cells of atherosclerotic lesions. Last year, we reported on platelet-mediated cholesterol accumulation in cultured aortic smooth muscle cells. In those studies, thrombin-activated washed platelets released a factor that, when added to cultured rat aortic smooth muscle cells, induced cholesteryl ester lipid droplet accumulation as detected histochemically. We have confirmed that this factor stimulates synthesis of cholesteryl ester in the smooth muscle cells.

We have also determined that platelets release large amounts of unesterified cholesterol when they are activated. Cholesterol release is calcium dependent and can be totally or partially blocked by a variety of anti-platelet drugs. The cholesterol that is released may be heterogeneous as indicated by a minor low density and a major high density fraction obtained using sucrose density gradient analysis. One possibility that we are exploring is whether the platelet factor that induces cellular cholesteryl ester accumulation is a cholesteryl-containing lipoprotein released from platelets when they are activated.

In collaboration with Dr. Marina Glukhova (Cardiology Research Institute, Moscow, USSR), we have demonstrated that platelet factor stimulates cholesteryl ester accumulation in a subpopulation of cultured human smooth muscle cells. This observation suggests that vascular smooth muscle cells may be comprised of at least two functional subtypes with respect to platelet-mediated cholesterol accumulation. Dr. Glukhova will attempt to clone the human vascular smooth muscle cells in order for us to carry out additional studies of this functional heterogeneity.

The possible role of platelets in promoting smooth muscle cell proliferation within atherosclerotic lesions has been previously recognized. Our research has now shown that platelets can also mediate cholesteryl ester accumulation within cultured vascular-derived smooth muscle cells. Our studies of platelet-mediated cholesterol accumulation will focus on the isolation and characterization of the platelet factor and the study of its interaction with cells associated with atherosclerotic lesions.

In collaboration with Dr. Wilbert Gamble (Oregon State University), we continued our characterization of cholesterol-rich lipid particles isolated from atherosclerotic vessels. Two major fractions of cholesterol-rich lipid

particles were isolated from atherosclerotic aortas; one fraction has a density less than 1.00 with cholesterol mostly in an esterified form. The other fraction has a mean density of approximately 1.02 with cholesterol mostly in an unesterified form. Interestingly, we have found that sphingomyelin is the predominant phospholipid in the unesterified cholesterol-rich fraction.

The identification of sphingomyelin as the predominant phospholipid associated with lesion unesterified cholesterol is an important new finding. From histochemical studies, we know that these unesterified cholesterol-rich particles occur within cells and in the extracellular space. These particles contain unesterified cholesterol that is less extractable than is normal plasma membrane unesterified cholesterol. Because large amounts of sphingomyelin also accumulate in atherosclerotic vessels, and considering cholesterol's high affinity for sphingomyelin, we now have a theoretical basis to help explain the retention of unesterified cholesterol in atherosclerotic lesions.

Work has been successfully completed on our project designed to develop a means of detecting and quantifying foam cells (i.e., cholesteryl ester-containing cells) in atherosclerotic vessels using flow cytometry and cell sorting. Single cell suspensions of aortic cells were prepared by enzymatic dissociation of aortas from swine with spontaneous and experimentally induced (using dietary cholesterol) atherosclerosis. Cellular cholesteryl ester was enzymatically hydrolyzed and specifically stained with the fluorescent dye filipin. The numbers of foam cells in each aorta was determined using flow cytometry.

Animals maintained on a cholesterol-free diet showed an increasingly variable number of foam cells as these animals increased in age. It was of interest that the atherosclerotic process appears to begin at a very early age in swine, as even some six-month-old animals had small numbers of foam cells. Some older animals had elevated numbers of foam cells, whereas other older animals had numbers of foam cells similar to levels found in younger 6-month-old animals. There was no statistical correlation of foam cell number and serum cholesterol level in the spontaneous disease group. A possible genetic basis for the variability in atherosclerosis among these animals was found. Although elevation of serum cholesterol induced by cholesterol feeding was associated with higher foam cell number, the correlation of serum cholesterol and foam cell number was not strong. Reversal of cholesterol feeding resulted in a reversal of the cholesterol-induced stimulation of foam cell formation.

Our findings suggest that swine may serve as an excellent animal model to examine those genetic and environmental factors contributing to atherosclerosis unrelated to dietary or plasma cholesterol. Our findings in swine are highly significant considering that plasma cholesterol levels do not explain the occurrence of most human atherosclerosis. We have shown that "foam cells" can be detected and quantified using flow cytometry. This highly sensitive assay provides a means to detect and quantify early atherosclerotic events. In addition, this assay may be useful in quantifying circulating "foam cells" providing a non-invasively derived index of the atherosclerotic process.

Work has been carried out in collaboration with other NHLBI laboratories over the past year. The relationship of atherosclerosis, lipoproteins, and coronary vasospasm was examined in collaboration with Dr. Dennis Sprecher (Molecular Disease Branch). Spectroscopic analysis of human aorta as an aid in laser selection for angioplasty was carried out in collaboration with Dr. Martin Leon (Cardiology Branch) and Dr. David Underhill (Cardiac Surgery Branch).

We have also over the past year collaborated with Dr. Peter Pentchev and Dr. Roscoe Brady of the Developmental and Metabolic Neurology Branch, NINCDS. Together, we have identified the metabolic abnormality in a mutant strain of BALB/c mice as a defect in the esterification of exogenous cholesterol. This leads to progressive accumulation of unesterified cholesterol within cells that resembles in many respects unesterified cholesterol accumulation in atherosclerotic lesions. Most importantly, we have determined that this same metabolic defect occurs in Type C Niemann-Pick disease. Our studies have also provided in vitro tests for diagnosing this disease.

In conclusion, significant progress has been made in the LEA towards our goal of defining the nature of accumulated cholesterol and the mechanism of cholesterol accumulation within atherosclerotic lesions. We have defined novel vessel-associated lipid particles that may play an important role in the regulation of arterial cholesterol metabolism. We have also shown how platelets may play an important role in causing cholesterol to deposit within vascular cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02824-06 EA

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Flow cytometric analysis of cells isolated from atherosclerotic lesions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Cupp	Staff Fellow	NHLBI, LEA
Others:	H.S. Kruth	Senior Investigator	NHLBI, LEA

COOPERATING UNITS *(if any)*
Perinatal Toxicology, Division of Toxicology, FDA (M.A. Khan and G.R. Henderson) Laboratory of Statistical and Mathematical Methodology, Division of Computer Research and Technology, NIH (G. Campbell), Lipid Nutrition Lab, USDA (E. Berlin)

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objective of this project was to develop a means of detecting and quantifying foam cells (i.e., cholesteryl ester-containing cells) in atherosclerotic vessels using flow cytometry and sorting. Single cell suspensions of aortic cells were prepared by enzymatic dissociation of aortas from swine with spontaneous and experimentally induced (using dietary cholesterol) atherosclerosis. Cellular cholesteryl ester was enzymatically hydrolyzed and specifically stained with the fluorescent dye filipin. The numbers of cholesteryl ester-containing cells in each aorta were determined using flow cytometry.

Animals maintained on a cholesterol-free diet showed an increasingly variable number of foam cells as these animals increased in age. It was of interest that the atherosclerotic process appears to begin at a very early age in swine, as even some six-month-old animals had small numbers of foam cells. Some older animals had elevated numbers of foam cells, whereas other older animals had numbers of foam cells similar to levels found in younger 6-month-old animals. There was no statistical correlation of foam cell number and serum cholesterol level in the spontaneous disease group. A possible genetic basis for the variability in atherosclerosis among these animals was found.

Although elevation of serum cholesterol induced by cholesterol feeding was associated with higher foam cell number, the correlation of serum cholesterol and foam cell number was not strong. Reversal of cholesterol feeding resulted in a reversal of the cholesterol-induced stimulation of foam cell formation.

We have shown that "foam cells" can be detected and quantified using flow cytometry. This highly sensitive assay provides a means to detect and quantify early atherosclerotic events. In addition, this assay may be useful in quantifying circulating "foam cells" providing a non-invasively derived index of the atherosclerotic process.

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Objective: The objective of this project was to develop a means of detecting and quantifying foam cells (i.e., cholesteryl ester-containing cells) in atherosclerotic vessels using flow cytometry and sorting.

Methods: See report of last year (Z01 HL 02824-05 EA) for detailed description of methods. In brief, single cell suspensions of aortic cells were prepared by enzymatic dissociation of aortas from swine with spontaneous and experimentally induced (using dietary cholesterol) atherosclerosis. Cellular cholesteryl ester was enzymatically hydrolyzed and specifically stained with the fluorescent dye filipin. The numbers of cholesteryl ester-containing cells (i.e., foam cells) in each aorta was determined using flow cytometry.

Major Findings: Animals maintained on a cholesterol-free diet showed an increasingly variable number of foam cells as these animals increased in age. The numbers of foam cells in the spontaneous disease group ranged from 0 to 412 per mm^2 of aortic surface area. It was of interest that the atherosclerotic process appears to begin at a very early age in swine. Even some six-month-old animals had small numbers of foam cells. Some older animals had elevated numbers of foam cells, whereas other older animals had numbers of foam cells similar to levels found in younger 6-month-old animals.

The variability of foam cell numbers in swine was not explained by variability in serum cholesterol levels and suggests that some swine are prone to the development of spontaneous atherosclerosis, whereas other swine are relatively resistant to the development of atherosclerosis. Preliminary analysis of the genetic background of these swine has shown that those animals with the highest numbers of foam cells had much greater commonality among ancestors than did animals with low numbers of foam cells; this suggests a possible genetic basis for the variability in atherosclerosis among these animals. Similar increases in atherosclerosis and variability of atherosclerotic disease with age have been observed in human populations.

The numbers of foam cells in cholesterol-fed animals with accelerated atherosclerosis ranged from 126 to 8446 per mm^2 of aortic surface area. The cholesterol-fed animal that had only 126 foam cells also had the lowest mean serum cholesterol value (215 mg%) over the six-month feeding period; however, this animal's serum cholesterol was not much less than the animal with the next lowest serum cholesterol level (225 mg%) but with substantially more foam cells (4396). Although elevation of serum cholesterol induced by cholesterol feeding was associated with higher foam cell number, the correlation of serum cholesterol and foam cell number was not strong (Kendall correlation, $\tau = .6$; $p = .09$). In addition, slight elevation of serum cholesterol from control levels of 55 to 115 mg% was not associated with an increase in aortic foam cell number suggesting that a cholesterol threshold may exist for stimulation of foam cell accumulation.

Significance to Biomedical Research and the Program of the Institute: We have shown that cholesteryl ester-containing cells (often referred to as "foam cells") can be detected and quantified using flow cytometry. This highly sensitive assay provides a means to detect and quantify early atherosclerotic events. In addition, this assay may be useful in quantifying circulating "foam cells" providing a non-invasively derived index of the atherosclerotic process.

In addition, our findings suggest that swine may serve as an excellent animal model to examine those genetic and environmental factors contributing to atherosclerosis unrelated to dietary or plasma cholesterol. Our findings in swine are highly significant considering that plasma cholesterol levels do not explain the occurrence of most human atherosclerosis.

Proposed Course: Project completed.

Publications:

1. Kruth, H.S., Cupp, J.E., Khan, M.A., Henderson, G.R., Berlin, E., and Campbell, G.: Quantification of cholesteryl ester-containing cells from swine aortas with spontaneous and experimentally induced atherosclerosis. Swine in Biomedical Research. New York, Alan R. Liss, Inc., In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HL 02826-04 EA

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Isolation and characterization of lipid-rich particles in atherosclerotic lesions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: H.S. Kruth Senior Investigator NHLBI, LEA

COOPERATING UNITS *(if any)*

Dept. Biochemistry & Biophysics, Oregon State University (W. Gamble), Lab of Cellular & Developmental Biology, NIADDK (M. Sliwkowski), Section on Lab Animal Medicine and Surgery, NHLBI (J. Pierce)

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Vascular Physiology Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to isolate and characterize unesterified cholesterol-rich lipid particles previously identified by us in atherosclerotic lesions using histochemical methods.

Two major fractions of cholesterol-rich lipid particles were isolated from atherosclerotic aortas; one fraction has a density less than 1.00 with cholesterol mostly in an esterified form. The other fraction has a mean density of approximately 1.02 with cholesterol mostly in an unesterified form. Interestingly, we have found that sphingomyelin is the predominant phospholipid in the unesterified cholesterol-rich fraction.

The identification of sphingomyelin as the predominant phospholipid associated with lesion unesterified cholesterol is an important new finding. From histochemical studies, we know that these unesterified cholesterol-rich particles occur within cells and in the extracellular space. These particles contain unesterified cholesterol that is less extractable than is normal plasma membrane unesterified cholesterol. Because large amounts of sphingomyelin also accumulate in atherosclerotic vessels, and considering cholesterol's high affinity for sphingomyelin, we now have a theoretical basis to help explain the retention of unesterified cholesterol in atherosclerotic lesions.

This finding contributes to our understanding of cholesterol deposition and will be important for the development of drugs designed to remove cholesterol from atherosclerotic lesions.

Objectives: The purpose of this project is to isolate and characterize unesterified cholesterol-rich lipid particles previously identified by us in atherosclerotic lesions using histochemical methods.

Methods: For detailed description of methods, see last year's report (Z01 HL 02826-03 EA). Briefly, normal and atherosclerotic rabbit aortas were homogenized, tissue fragments were removed by centrifugation and filtration, and lipid particles were isolated by floatation on a sucrose density gradient. Lipid particles were analyzed using standard techniques of protein and lipid analysis.

Major Findings: Two major fractions of cholesterol-rich lipid particles were isolated from atherosclerotic aortas; one fraction has a density less than 1.00 with cholesterol mostly in an esterified form. The other fraction has a mean density of approximately 1.02 with cholesterol mostly in an unesterified form. This fraction is heterogeneous morphologically containing rod-shaped crystalline structures and spherical lipid particles. The major protein associated with the unesterified cholesterol-rich fraction is albumin, whereas little protein was associated with the cholesteryl ester-rich fraction. Interestingly, we have found that the unesterified cholesterol-rich fraction is rich in sphingomyelin, the predominant phospholipid in this fraction. We believe that the unesterified cholesterol-, sphingomyelin-rich fraction corresponds to the unesterified cholesterol-rich lipid particles that we identified previously in atherosclerotic lesions using histochemical methods.

Significance to Biomedical Research and the Program of the Institute: We have successfully isolated unique lipid particles from atherosclerotic aortas that are rich in unesterified cholesterol. From our previous histochemical studies, we know that these unesterified cholesterol-rich particles occur within cells and in the extracellular space and contain unesterified cholesterol that is less extractable than is normal plasma membrane unesterified cholesterol. The identification of sphingomyelin as the predominant phospholipid associated with lesion unesterified cholesterol is an important new finding. Because large amounts of sphingomyelin also accumulate in atherosclerotic vessels, and considering cholesterol's high affinity for sphingomyelin, we have a theoretical basis to explain the retention of unesterified cholesterol in atherosclerotic lesions. This finding contributes to our understanding of cholesterol deposition and will be important for the development of drugs designed to remove cholesterol from atherosclerotic lesions. Our observations concerning unesterified cholesterol-, sphingomyelin-rich lipid particles takes on added importance in view of our recently reported findings that deposition of unesterified cholesterol is the earliest detectable form of accumulated lipid in developing atherosclerotic lesions.

Proposed Course: Studies will be carried out to further fractionate and additionally characterize the unesterified cholesterol-rich lipid particles isolated from atherosclerotic vessels.

Publications:

1. Kruth, H.S.: Lipid deposition in human tendon xanthoma. Am. J. Path., In press.

2. Kruth, H.S.: Subendothelial accumulation of unesterified cholesterol - an early event in atherosclerotic lesion development. Atherosclerosis., In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02827-03 EA

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Platelet-mediated cholesterol accumulation within vascular-associated cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: H.S. Kruth Senior Investigator NHLBI, LEA

COOPERATING UNITS *(if any)*

Developmental and Metabolic Neurology Branch, NINCDS (P. Pentchev), Department of Biochem. & Biophys., Oregon State University (W. Gamble), Institute of Experimental Cardiology, Cardiology Research Institute, Moscow, USSR (M. Glukhova)

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Vascular Physiology Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The purpose of this project is to examine mechanisms of cellular cholesteryl ester accumulation that may be mediated by platelets. Washed rat platelets have been incubated with smooth muscle cells, macrophages, fibroblasts, and endothelial cells. In addition, platelet-free supernatants, prepared from unactivated or activated incubated platelets have been added to cultures of these cells.

As reported last year (Z01 HL 02827-02 EA), activated rat platelets induce cholesteryl ester accumulation in cultured rat aortic smooth muscle cells. We have determined this year that platelet activation by collagen is equally effective as is platelet activation with thrombin. Histochemical detection of smooth muscle cholesteryl ester accumulation has been confirmed chemically as platelet-mediated stimulation of cholesteryl ester synthesis. Interestingly, cholesteryl ester accumulates in a subpopulation of cultured human aortic smooth muscle cells, suggesting that smooth muscle cells may be comprised of at least two functional subtypes with respect to platelet-mediated cholesterol accumulation.

We have also determined that platelets release substantial amounts of unesterified cholesterol when they are activated. Cholesterol release is calcium dependent and can be totally or partially blocked by a variety of anti-platelet drugs.

The possible role of platelets in promoting smooth muscle cell proliferation within atherosclerotic lesions has been previously recognized. Our research has shown that platelets can mediate cholesteryl ester accumulation within cultured vascular-derived smooth muscle cells. The possible role of platelets in mediating cholesterol accumulation within atherosclerotic lesions must now also be considered in evaluating the pathogenesis of atherosclerosis.

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Objectives: The purpose of this project is to examine mechanisms of cellular cholesteryl ester accumulation that may be mediated by platelets.

Methods: Washed rat platelets have been added to cultured cells including smooth muscle cells, macrophages, fibroblasts, and endothelial cells. Platelets have been co-cultured with these cells without and with thrombin or collagen activation of the added platelets. In addition, platelet-free supernatants, prepared from unactivated or activated incubated platelets have been added to cell cultures. Cellular cholesteryl ester accumulation was monitored using filipin to histochemically detect cholesteryl ester lipid droplets, and using ^{3}H-oleate to quantify cholesteryl ester synthesis.

To determine whether platelets release cholesterol that could be a possible source of cellular cholesteryl ester, cholesterol was quantified in platelet-free supernatants prepared from incubated unactivated and activated platelets.

Major Findings: As reported last year (Z01 HL 02827-02 EA), activated rat platelets induce cholesteryl ester accumulation in cultured rat aortic smooth muscle cells. We have determined this year that platelet activation by collagen is equally effective as is platelet activation with thrombin. Histochemical detection of smooth muscle cholesteryl ester accumulation has been confirmed chemically as platelet-mediated stimulation of cholesteryl ester synthesis.

Interestingly, cholesteryl ester accumulates in one human aortic smooth muscle cell line and in rat peritoneal macrophages, even when unactivated washed platelets are added to these cell cultures. Supernatants prepared from activated washed platelets induce cholesteryl ester accumulation in a subpopulation of the cultured human smooth muscle cells. The latter observation suggests that vascular smooth muscle cells may be comprised of at least two functional subtypes with respect to platelet-mediated cholesterol accumulation.

We have determined that activated platelets release substantial amounts of unesterified cholesterol when they are activated. Cholesterol release is calcium dependent and can be totally or partially blocked by a variety of anti-platelet drugs. The cholesterol that is released may be heterogeneous as indicated by a minor low density and a major high density fraction obtained using sucrose density gradient analysis.

Significance to Biomedical Research and the Program of the Institute: The possible role of platelets in promoting smooth muscle cell proliferation within atherosclerotic lesions has been previously recognized. Our research has shown that platelets can mediate cholesteryl ester accumulation within cultured vascular-derived smooth muscle cells. The possible role of platelets in mediating cholesterol accumulation within atherosclerotic lesions must now also be considered in evaluating the pathogenesis of atherosclerosis.

Atherosclerosis is the underlying basis of most coronary artery disease, the leading cause of death in the United States. Because pathological cellular cholesterol accumulation is central to the atherosclerotic process, our new findings concerning the role of platelets in mediating cellular cholesterol accumulation are of great significance to the program of the NHLBI.

Proposed Course: Studies will be carried out to isolate and characterize the factor released by activated platelets that induces cholesteryl ester accumulation in vascular smooth muscle cells.

Publications:

1. Kruth, H.S.: Platelet-mediated cholesterol accumulation in cultured aortic smooth muscle cells. Science 227:1243-1245, 1985.

2. Kruth, H.S.: Cholesterol accumulation in vascular smooth muscle cells incorporated into platelet-rich plasma clots. Lab Invest., In press.

ANNUAL REPORT OF THE
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1984 through September 30, 1985
Section of Experimental Therapeutics

This year the Experimental Therapeutics Section of the Hypertension-Endocrine Branch has continued its basic and clinical research into many aspects of the causes and therapy of hypertension. These studies have focused on the neurohumoral and vasoactive systems that control the circulation.

I. Catecholamines and the sympathetic nervous system. Our previous studies indicated that about 20% of patients with essential hypertension have high plasma levels of norepinephrine (NE). We therefore administered clonidine, a drug that blocks central sympathetic outflow, to 44 patients with essential hypertension and 41 normal control subjects of similar age to determine if the level of NE indicated the contribution of the sympathetic nervous system to the hypertension. In the patients with essential hypertension, the resting level of plasma NE was significantly related to the decrease in mean arterial pressure three hours after a single dose of clonidine. The magnitude of the depressor response in the patients also was correlated significantly with the decrease in plasma NE after clonidine. These results suggest that increased sympathetic outflow plays a pathophysiologic role in some patients with essential hypertension, and that the combination of a high baseline NE with an excessive depressor response to clonidine identifies patients with neurogenic hypertension. To measure more accurately the activity in the sympathetic nervous system, we developed techniques to estimate the cleft-plasma concentration gradient and regional neuronal removal of NE. We did this by measuring arterial NE during NE infusions and during yohimbine-induced release of endogenous NE in subjects pretreated with desipramine. We could then calculate the cleft-NE concentration associated with a 20 mmHg sympathetically mediated pressor response. During NE infusions in healthy subjects, linear pressor-log NE concentration relationships were observed. At a pressor response of 20 mmHg, arterial NE averaged 3,647 pg/ml. The pressor-log NE relationship was shifted more than five-fold to the left during combined ganglionic, alpha-2 adrenoreceptor, and uptake-1 blockade (arterial NE = 684 pg/ml). During yohimbine-induced release of endogenous NE in desipramine-pretreated subjects, arterial NE averaged 467 pg/ml. Since the concentration of NE in the synaptic clefts must have been between the values for plasma NE during its infusion and during its endogenous release, we therefore estimated that in healthy people, a 20 mmHg sympathetically mediated pressor response is associated with about a 560 pg/ml concentration of NE in the average neuroeffector junction. This is the first time such a measurement has been made in man and the meam value obtained from our 18 healthy volunteers is essentially the same as that obtained from a similar set of experiments in rats. These results may now be compared with those of patients with essential hypertension, in order to separate excessive cleft NE concentrations from excessive vascular responsiveness to endogenous NE as determinants of pressor hyper-responsiveness in essential hypertension. By infusing simultaneously tracer-labeled NE and tracer-labeled isoproterenol, which is not a substrate for neuronal uptake, and sampling arterial and venous blood at the study state, regional, total, and neuronal removal of NE could be quantified. When this was done, we found that the arm removes about 50% of arterial NE. Pretreatment with desipramine decreased the proportionate NE

removal in the arm to exactly that of isoproterenol, indicating that the difference in removal of NE and isoproterenol could be accounted for by uptake-1. We estimated that about 15% of infused NE which is removed in the arm, is removed by uptake-1. These results may also be compared with those in patients with essential hypertension, some of whom may have defective uptake-1 due to a circulating inhibitor of Na/K ATPase. We also gave intravenous infusions of isoproterenol to detect possible abnormalities of presynaptic beta-adrenoreceptor function in hypertension. The isoproterenol infusions produced dose-related increments in heart rate and cardiac output with little effect on mean arterial pressure, and with decreased total peripheral resistance as measured by impedance cardiography. Plasma NE increased in a dose-related manner in all the subjects. Plasma levels of epinephrine were uneffected. The results support the hypothesis that stimulation of presynaptic beta adrenoceptors causes release of endogenous NE from sympathetic nerve endings, but does not affect adrenomedullary secretion of epinephrine. The results also indicate that epinephrine is not co-released from sympathetic nerve endings and that isoproterenol can indirectly enhance alpha 1 adrenoreceptor-mediated vasoconstriction by stimulating release of endogenous NE. To determine the possible role of circulating dopamine, dopamine was infused into healthy men in amounts to produce three different study state plasma dopamine concentrations of 0.7, 3.7, and 38 ng/ml. Mean dopamine excretion increased significantly from basal levels during all three dopamine infusions. NE excretion increased during both the medium and high dose infusions, whereas sodium excretion, plasma NE and heart rate increased only during the high dose dopamine infusion. Basal aldosterone values were low and did not change. Since a natriuretic response occurred only at supraphysiologic concentrations of circulating dopamine, if dopamine has any physiologic role, it must be released from dopaminergic neurons or otherwise produced locally in very high concentrations within the kidney. We examined arterial and venous blood samples in three patients who had undergone regional sympathectomies and found that in the sympathectomized limbs, arterial NE exceeded venous by about 40%. This is opposite to results in normal subjects and in intact limbs of these same patients, where venous NE exceeded arterial NE. These results show that the arterial venous increment usually seen in plasma NE depends upon local release from sympathetic nerve endings. The presence of an arteriovenous decrement in plasma NE establishes sympathetic denervation. In general, the results confirm the validity of plasma levels of NE as indicators of sympathetically mediated NE release.

In collaborative studies with J.S. Turkkan, we have shown that administration of sodium chloride to baboons has little effect on either systolic or diastolic blood pressure, regardless of the route of administration of the salt. However, salt loading combined with DOCA rapidly produced persistent increases in pressure which averaged 20-30 mmHg. DOCA/salt hypertension was associated with increased water intake, decreased plasma potassium, and decreased renin activity, but there were no consistent changes in plasma catecholamines or heart rate. When clonidine, hydrochlorothiazide with triamterene, and atenolol were tested for their antihypertensive effects during DOCA/salt hypertension, only the diuretic combination affected blood pressure. This is the first report of DOCA/salt hypertension in primates, and the results indicate that in this low-renin hypertensive model, increased sympathetic nervous activity does <u>not</u> play an important role. In another study six of nine baboons who underwent diastolic blood pressure conditioning had progressive, significant increases in resting, inter-session averaged pressure. Two animals

had nephrosclerosis and one had fibrinoid vascular necrosis. Primates experiencing repeated, conditioned pressor episodes can develop increases in resting blood pressure, but chronically repeated pressor episodes do not invariably lead to sustained hypertension. These results are consistent with the hypothesis that episodes of high blood pressure cause structural adaptation of vascular walls only in predisposed individuals.

In previous studies, we had observed the pattern of plasma NE and epinephrine responses to third molar extractions. We had found that significant increases in circulating epinephrine levels and cardiac output were seen in unsedated patients after administration of lidocaine with epinephrine before third molar extractions, whereas no changes were seen after lidocaine alone. Unsedated patients had increased NE and epinephrine levels during the surgery. Sedation with diazepam decreased NE levels below the preoperative values, followed by increases during surgery to preoperative levels. Diazepam abolished the epinephrine response to the surgery, whereas the NE responses were unaffected. The results indicate that intra-oral injection of an epinephrine-containing local anesthetic results in increased circulating epinephrine levels that are associated with cardiovascular stimulation. Diazepam directly inhibits sympathoadrenomedullary responses to surgical stress. We also found that endogenous opioids increase during the stress independent of the catecholamine responses.

We have developed several measurement techniques to study the pathophysiology of hypertension. We have compared values for cardiac output, using the non-invasive impedance cardiography technique with values obtained using an invasive technique mainly thermodilution, and also assessed hemodynamic responses to manipulations which differentially affect heart rate and stroke volume. In both situations there was a correlation of 0.86 and a p value <0.001. Thus impedance cardiography appears to measure validly cardiac output and stroke volume in man. We are currently making use of ultrasonic Doppler techniques for measuring blood flow velocity in the forearm to determine forearm blood flow and vascular distensibility. We seek to do this because our studies of the brachial arterial dicrotic wave in response to vasoconstrictors or vasodilators have shown that in hypertensive patients the vertical modulation is defective. The computer modelling of this situation indicates that in early hypertension vascular distensibility is decreased. The Doppler method for measuring blood flow velocity appears to work well. However, the ultrasonic method for measuring changes in vascular caliber poses significant problems that we are currently attempting to overcome. Laser-Doppler flowmetry provides a continuous measurement of skin microvascular blood flow. We used this technique in patients who had undergone sympathectomies in order to assess the completeness of sympathetic denervation and to determine whether spontaneous oscillations of microvascular flow result from fluctuations in sympathetic activity. Indeed, no microvascular reflexive responses were observed in sympathectomized limbs. Spontaneous oscillations of microvascular flow, however, were prominent even in sympathectomized limbs, indicating that the oscillations do not depend on spontaneous changes in sympathetic microvascular tone. The spontaneous oscillations could be eliminated by systemic doses of nifedipine, a calcium channel blocker with potent peripheral vasodilator action.

II. <u>The role of salt in hypertension.</u> In our previous studies of

patients with normal renin essential hypertension, those that were salt resistant were shown to have higher urinary dopamine than those that were salt sensitive. The salt resistant patients also suppressed their plasma NE in response to a high sodium intake, whereas the salt sensitive patients did not. Similar studies performed in normal subjects indicate that when sodium intake was increased from 9 to 249 mEq/day, sodium retention was only slightly less than that in the salt resistant patients, but significantly less than that in the salt sensitive patients. Urinary dopamine did not differ from that in the salt sensitive patients, but was significantly lower than that in the salt resistant patients. Plasma NE also did not differ from that in the salt sensitive patients, but was significantly higher than that in the salt resistant patients who suppressed plasma NE in response to high salt intake. These results suggest that during a high salt intake, supranormal renal dopaminergic activity in the salt resistant hypertensives prevents supranormal sodium retention; the dopaminergic nervous system may also mediate the supranormal decrease in plasma norepinephrine in these patients. Supranormal sodium retention that does not suppress plasma NE may mediate, in part, the increase in blood pressure that occurs during a high sodium intake in the salt sensitive hypertensives. These results suggest that patients with normal renin essential hypertension may have a genetically increased renal reabsorption of sodium chloride. It will be important to see if such salt sensitivity occurs in normotensive offspring of hypertensive parents.

III. Role of calcium in hypertension. Recent reports by other investigators have suggested that a subnormal intake of calcium and/or a supranormal intracellular level of calcium may cause hypertension. We therefore set up and validated the Quin-2 method for measurement of intracellular free calcium in our laboratory. We then measured basal free intracellular calcium levels in platelets from both spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKR). There were no differences in either the basal free intracellular calcium levels or in intracellular calcium levels during calcium depletion, repletion, or during stimulation with thrombin, ADP, or serotonin. Thus, at least in this model for hypertension, there are no apparent differences in intracellular free calcium. Recently, Blaustein has hypothesized that levels of intracellular calcium are elevated in hypertension due to stimulation of a transmembrane sodium-calcium exchange mechanism by a circulating inhibitor of the sodium-potassium ATPase. To test this hypothesis, we examined intracellular calcium levels in lymphocytes, platelets, and adrenal medullary cells during incubations with different concentrations of ouabain, an inhibitor of Na-K/ATPase. When calcium was added to calcium-depleted cells, ouabain incubation caused faster initial influx of calcium, but intracellular free calcium rapidly returned to control levels. These data indicate that the rapid and effective calcium buffering mechanisms in cells prevent changes in free intracellular calcium levels and refute Blaustein's hypothesis. To study further the role of calcium in catecholamine release, we set up cultures of bovine adrenal medullary cells. We showed that basal levels of intracellular calcium were increased rapidly by stimulation with nicotine, high potassium, and veratridine, and that these increases were associated with rapid increases in the rate of 45 Ca influx. The pattern of changes in intracellular calcium and the rate of return to basal values after stimulation were different for the different secretagogues. Thus, veratridine caused a relatively small increase in intracellular calcium; however the amount of catecholamine released was the largest. A calcium ionophore increased intracellular calcium, but did not

affect catecholamine release. There was no increase in intracellular calcium and no additional catecholamine release upon a second stimulation with nicotine. However, high potassium stimulation after nicotine produced further increases in intracellular calcium and in catecholamine release. Repetitive stimulation with high potassium yields repetitive increases in intracellular calcium and in catecholamine release, while high sodium in the media inhibited the effect of high potassium, but not the effect of nicotine, on calcium levels in the cells. We believe that these data show that the rate of catecholamine release correlates best with the rate of calcium influx and less well with the absolute level of intracellular calcium. In certain circumstances, increases in intracellular calcium were not accompanied by any catecholamine release. Our data suggest that the presence of calcium within specific calcium channels may be the factor which initiates the secretory process. It is clear that nicotine and high potassium affect two different channels through which calcium enters the cell.

IV. Atrial natriuretic peptides. We have been successful in generating an antiserum against alpha-rat ANP_{5-28} (ATP III). This antiserum has excellent cross reactivity with many atrial natriuretic peptides including Auriculin B, Auriculin A, ATP III, ATP II, and ATP I. It recognizes alpha-RANP $_{1-28}$ (ANF) and alpha-human ANP_{1-28} only 10% and <0.2% as well as ATP III, respectively. With use of this antibody and liquid chromatographic separation techniques, we have shown that the major circulating forms of ANP in the rat are alpha-rat ANPs-28 and alpha-rat ANP 3-28. IR-ANP levels in conscious, unrestrained control animals with indwelling catheters were found to be in the range of 80-120 pg/ml. Ours was the first report of IR-ANP levels in non-anesthetized, non-traumatized animals and our values were generally 1/5-1/15 of previously reported values. Volume loading (VL) with either 5% glucose or 0.9% saline enhanced plasma levels of IR-ANP 4-5 fold. Hyperosmotic challenge also resulted in a rapid increase in plasma ANP similar to that observed following volume loading. Halothane anesthesia resulted in 3-fold increases in plasma IR-ANP. Vagotomy in halothane-anesthetized rats or pentobarbital anesthesia did not alter circulating ANP levels or modify the VL-induced release of ANP. Complete cardiac denervation in the pithed-rat preparation, which removes both humoral influences of central nervous system origin and direct neuronal control of the heart by both the vagal and sympathetic nerves, blocked the VL induced release of ANP. We next infused rat ATP III into conscious, instrumented SHR and WKR. There was a fall in mean arterial pressure in both strains of approximately 22%, which was due predominantly to a decrease in cardiac index, secondary to a fall in stroke volume. This was associated with a significant increase in peripheral resistance. Plasma catecholamines and heart rate were not significantly altered. Maximum increases in cardiac index and stroke volume, and the fall in peripheral resistance after VL were unaffected by the ATP III. In addition, although the plasma levels of ANP obtained with the 2 ug/kg bolus dose were comparable with those noted with volume expansion alone, the hemodynamic responses were completely different. Bolus injection of ANP decreased both cardiac index and peripheral resistance, while volume loading increased cardiac index and decreased peripheral resistance. One explanation for these data would be that ANP had a negative ionotrophic effect on the heart. However, when ANP was introduced into the Langendorf isolated heart preparation, there was no change in either heart rate or strength of contraction. Thus the reduction in blood pressure induced by ANP must be due to a decrease in preload, and not due to any change in myocardial function or afterload. Direct measurement of a

decrease in preload is very difficult in small animals for technical reasons.

Administration of ANP increased urinary sodium excretion in 4 of 5 dogs studied. There were no changes noted in renal blood flow, glomerular filtration, or left ventricular diastolic pressure during the ANP-induced natriuresis. No change was seen in renal arterial or venous DOPA or NE levels following ANP infusion, and dopamine excretion was also unchanged. These results suggest that the renal production of dopamine is not involved in the action of ANP in increasing renal excretion of sodium and decreasing renal release of renin.

Acute pressor responses to bolus injected clonidine in pithed rats were dose-dependently attenuated by ANP and nifedipine, but acute pressor responses to phenylephrine were unaffected. Sodium nitroprusside equally inhibited pressor responses to clonidine and phenylephrine. Pressor responses during constant infusions of clonidine and phenylephrine were attenuated similarly by ANP and nifedipine. This pattern of results -- alpha-2 adrenoreceptor specificity during immediate pressor responses, but not during sustained pressor responses -- suggests that ANP, like nifedipine, attenuates those adrenoreceptor-mediated responses which depend on slow transmembrane calcium fluxes. Further work on these mechanisms of action of ANP is important since the actual mechanism for the antihypertensive effect of ANP has not been elucidated.

V. Renin-angiotensin system. The role of the renin-angiotensin system in the renal vasoconstriction produced by acetylcholine in indomethacin-treated dogs was examined in dogs receiving infusions of saralasin, an angiotensin II receptor blocker, phenoxybenzamine, and propranolol. Infusion of acetylcholine into a renal artery of the dog causes an increase in renal blood flow and sodium excretion. We have shown previously that the synthesis of prostaglandins is required for the vasodilatory and natriuretic response to acetylcholine. In indomethacin-treated dogs receiving an infusion of saralasin alone, or in combination with alpha- and beta-blockade, renal arterial infusion of acetylcholine produced an initial rise in sodium excretion and renal plasma flow, with no change in renin secretion. This was followed by a fall in urinary sodium excretion and renal plasma flow, with an increase in renin secretion. Thus it appears that acetylcholine produces renal vasoconstriction in indomethacin-treated dogs by mechanisms other than an increase in the activity of either the renin-angiotensin system or the sympathetic nervous system.

VI. Malignant pheochromocytoma. Since malignant pheochromocytoma is a very rare tumor, there are no guidelines for chemotherapy. Last year we began a combination chemotherapeutic regimen for this disease based on the treatment of malignant neuroblastoma, a much more common tumor also of neural chest origin. The regimen consisted of cyclophosphamide, vincristine, and dacarbazine given in repeated cycles, each 21 days. We now have data on 8 patients who have been treated for periods of 4-20 months with this regimen. The 3 patients treated the longest, i.e., 10-20 months, have shown significant partial remissions with marked improvement in their performance status and in their sense of well being. The other 5 patients have shown some improvement, but their duration of therapy has so far been too short to allow adequate evaluation. Two of the original 3 patients have broken through the primary regimen after periods of 10 and 20 months respectively and have been placed on

other drugs. It is thus apparent that while significant partial remissions can be produced by this regimen, we cannot effect cures with it. It would appear that we will only get one chance at palliation with this regimen and that a significant but limited partial remission will be obtained. Thus we will limit therapy with this regimen to patients who have disseminated disease which is producing significant reductions in the quality of life and which can no longer be treated with other techniques. While this may seem to be somewhat disappointing, and it is, this is the first time that any chemotherapeutic regimen has been demonstrated to be at all effective in the treatment of malignant pheochromocytoma.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01965-02 HE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical methods for vasoactive substances

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	R. Stull	Chemist	HE, NHLBI
	J. Tate	Biol. Lab. Tech.	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have established methodology for measuring plasma, urine, and cerebrospinal fluid levels of DOPA, DOPAC, and the catecholamines using liquid chromatography with electrochemical detection. One source of contaminating peaks in the assay is dihydrocaffeic acid, a metabolite of caffeic acid, which is a catechol found in coffee. DOPA and DOPAC levels were increased in some patients with pheochromocytoma and in all of 5 patients with neuroblastoma. We currently are developing a liquid chromatographic-radioimmunoassay (LC-RIA) for atrial natriuretic factor.

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Objectives:

We are using liquid chromatography with electrochemical detection for measuring levels of DOPA, DOPAC, and the catecholamines, all of which may participate in neural circulatory control. We are developing LC-RIA methodology for the 1-28 form of human atrial natriuretic factor (ANF), which appears to be a natriuretic, cardiac hormone involved with volume homeostasis.

Methods:

DOPA, DOPAC, and the catecholamines are separated using liquid chromatography after a batch alumina extraction and quantified by the current produced upon exposure of the column effluent to an oxidizing potential. ANF is separated using gradient liquid chromatography after sample preparation using a C-18 minicolumn. The peak corresponding to the 1-28 form of ANF is subjected to an RIA.

Results and Their Significance:

Normal values for plasma DOPA and DOPAC were approximately 2000 pg/ml. DOPA and especially DOPAC were not recovered from the alumina extraction step as well as the internal standard, N-methyldopamine. Of 10 patients with pheochromocytoma, 4 had venous plasma DOPA levels exceeding 3 times the normal value. In contrast, all 10 of the patients with pheochromocytoma and venous plasma NE levels exceeding 10 times the normal value, whereas in 3 of the 5 patients with neuroblastoma, venous plasma NE levels were within the normal range. The results suggest that DOPA and DOPAC may be produced in tumors of neural crest origin and be released into the bloodstream. Plasma levels of DOPA and DOPAC may indicate the presence of neuroblastoma, especially if plasma levels of NE are in the normal range.

A contaminating peak occasionally co-eluted with epinephrine or dopamine during LC with electrochemical detection for plasma catecholamines. This peak was not present in subjects who had abstained from drinking coffee overnight, but it persisted in subjects who switched to decaffeinated coffee. Since the unknown substance was retained on alkaline alumina and desorbed from acid alumina, we reasoned that it was a catechol related to coffee drinking but unrelated specifically to caffeine, which is not retained on alumina. Dihydrocaffeic acid, a catechol metabolite of caffeic acid, co-eluted with the unknown substance. GC-MS positively identified the substance as dihydrocaffeic acid. Blood samples for plasma catecholamines should be drawn when the subjects have refrained from drinking coffee or even decaffeinated coffee for at least 18 hrs.

Proposed Course of Study:

Several experiments are in progress to determine the sources of circulating DOPA and DOPAC. Patients with pheochromocytoma and

neuroblastoma will be studied to determine if DOPA and DOPAC levels provide diagnostic information. The ANF methods development will continue.

References:

Shoup RE, Kissinger PT, Goldstein DS. Rapid liquid chromatographic methods for assay of norepinephrine, epinephrine, and dopamine in biological fluids and tissues. In Ziegler MG, Lake CR (Eds), Norepinephrine, Baltimore: Williams & Wilkins, 1984, pp. 38-46.

Goldstein DS, Stull R, Markey SP, Marks E, Keiser HR. Dihydrocaffeic acid: A common contaminant in the liquid chromatographic-electrochemical measurement of plasma catecholamines in man. J Chromatog 311:148-153, 1984.

Goldstein DS. Sample handling and preparation for liquid chromatographic-electrochemical assays for plasma catecholamines. In Krstulovic AM (Ed), Quantitative Analysis of Catecholamines. New York: Wiley Interscience (in press).

Goldstein DS, McDonald RF. Biochemical indices of cardiovascular reactivity. In Matthews K, Weiss S (Eds), Stress, Reactivity, and Cardiovascular Disease. New York: Wiley (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HL 01968-02 HE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Collaborative studies of neuroendocrine circulatory control.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	R. Udelsman	Medical Staff Fellow	SB, NCI
	K.M. Hargreaves	Postdoctoral Fellow	NAB, NIDR
	R. Dionne	Pharmacologist	NAB, NIDR
	M. Haass	Visiting Fellow	NIB, NINCDS
	Z. Zukowska-Grojec	Guest Researcher	OD, NINCDS
	I.J. Kopin	Scientific Director	OD, NINCDS
	R.S. Rittmaster	Medical Staff Fellow	DEB, NICHD
	R. Zimlichman	Visiting Associate	HE, NHLBI
	R. Stull	Chemist	HE, NHLBI

COOPERATING UNITS *(if any)*

Div. of Behavioral Biol., Johns Hopkins Sch. Med., Baltimore, MD (J.S. Turkkan)
Neurobiology Research Div., USUHS, Bethesda, MD (G.Z. Feuerstein)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have collaborated with several other groups in studies of neuroendocrine control of the circulation. We developed the first primate model for DOCA/salt hypertension. This form of hypertension is not associated with elevated levels of plasma catecholamines, and it is insensitive to adrenergic blockers but sensitive to diuretics. Chronically repeated pressor episodes resulting from operant diastolic blood pressure conditioning only inconsistently produced sustained increases in blood pressure in baboons. We found evidence for functional corticotropin-releasing hormone (CRF) receptors on adrenomedullary and sympathetic ganglionic cells. In human subjects who received ovine CRF, morphine blunted the ACTH response to CRF without affecting plasma catecholamines or vasopressin. In subjects undergoing third molar extractions, larger increases in plasma epinephrine than norepinephrine (NE) were observed. Increases in immunoreactive beta-endorphin during the surgery were blocked by fentanyl without affecting the catecholamine response. Administration of the mycotoxin, T-2, produces circulatory shock, associated with high catecholamine levels, apparently due to lactic acidosis. Intrarenal infusion of atrial natriuretic factor (ANF) did not affect urinary norepinephrine or dopamine production in dogs. ANF appears to inhibit differentially alpha adrenoceptor subtypes.

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Objectives:

Our group has collaborated with others in studies involving neuroendocrine circulatory controls. In collaboration with Dr. Turkkan, of the Johns Hopkins School of Medicine, we determined whether chronic repetition of pressor episodes eventually produces sustained hypertension, as would be predicted if structural vascular adaptation occurs in response to episodic hypertension from any source.

In collaboration with Dr. Turkkan, we also developed the first primate model of DOCA/salt hypertension. We measured plasma catecholamines to elucidate the mechanism of the hypertension, and we compared the efficacy of sympathetic blockers and diuretics in this experimental model.

CRF may participate in the organism's adaptation to stress. In collaboration with Dr. Udelsman, of the NCI, we determined whether functional CRF receptors exist on adrenomedullary cells. In collaboration with Dr. Rittmaster, of the NICHD, we determined whether morphine-induced attenuation of the ACTH response to CRF depends on effects of morphine on circulating catecholamines.

In collaboration with Dr. Hargreaves and Dr. Dionne, of the NIDR, we observed the pattern of plasma NE and epinephrine responses to third molar extractions and the effects of diazepam or fentanyl on the catecholamine and beta-endorphin responses to the surgery.

In collaboration with Dr. Feuerstein, of the USUHS, we determined whether T-2 toxin causes shock by sympathetic destruction.

In collaboration with Dr. Sowers, of the University of Virginia, we studied the mechanism of the natriuretic response to ANF, by measuring the effects of intra-renal infusion of ANF on urinary dopamine excretion in dogs. In collaboration with Dr. Zukowska-Grojec, responses to alpha adrenoceptor stimulation depends on the alpha-adrenoceptor subtype.

Methods:

Seven male baboons were chronically instrumented for direct, continuous recording of arterial pressure in order to study the effects of salt administration alone at two doses (8.6 and 17.1 mEq Na/kg/day of 5% sodium chloride, with four different routes of administration) and in combination with DOCA (5 mg/2 days im).

Nine male baboons with indwelling arterial catheters underwent operant diastolic blood pressure condition. All the animals had large-magnitude (mean 25 mm Hg) elevations in diastolic blood pressure during 12-hour training sessions which were repeated daily, usually for several months. Trends in blood pressure during the inter-session intervals were recorded.

CRF receptors were measured by binding of I-125-Tyr-CRF to the 30,000 g membrane-rich factions of tissue homogenates of adrenal glands and lumbar,

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chronically repeated pressor episodes do not invariably lead to sustained hypertension. The results are consistent with the hypothesis that episodes of high blood pressure cause structural adaptation of vascular walls only in predisposed individuals.

No CRF binding was found in the adrenal cortex, but saturable, high-affinity binding was present in adrenal meduallary tissue and confirmed by autoradiography. Specific CRF binding also was demonstrated in the lumbar, thoracic, cervical, and celiac sympathetic ganglia. CRF caused a small but significant and dose-dependent stimulation of adenylate cyclase activity in adrenal medullary cells. In cultured bovine adrenal medullary cells, short-term incubation with CRF (10-30 mins) had no effect on catecholamine secretion or intracellular calcium, but culture of the cells in the presence of CRF for 24 hrs resulted in significantly enhanced catecholamine release and increased intracellular calcium. Similar stimulation of release of met-enkephalin was observed upon incubation of the chromaffin cells with CRF for 24 hrs. The properties of the binding sites for CRF in the adrenal medulla, with specificity for CRF-related peptides, high affinity, and the ability to stimulate adenylate cyclase and the secretion of catecholamines and met-enkephalin, indicate that they represent functional receptors through which CRF modulates adrenal medullary function. The presence of similar binding sites in sympathetic ganglia suggests that CRF is also involved in the extra-adrenal peripheral autonomic nervous system.

Morphine blunted the ACTH response of humans for the first 60 mins and cortisol response for the first 90 mins after CRF administration. Morphine did not lower arginine vasopressin or catecholamine levels. Morphine may modulate the pituitary ACTH response to CRF through suprapituitary factors.

During third molar extractions, immunoreactive beta-endorphin increased. This increase was attenuated by fentanyl and enhanced by naloxone, whereas the NE responses were unaffected by these medications. The results indicate that intraoral injection of an epinephrine-containing local anesthetic results in increased circulating epinephrine levels that are associated with cardiovascular stimulation; that diazepam directly inhibits sympathetic activity and sympathoadrenomedullary responses to surgical stress; and that endogenous opioids increase during the stress independent of the catecholamine responses.

Injection of T-2 into conscious rats produced prolonged hypertension and tachycardia, followed by hypotension. Total peripheral resistance increased and cardiac output decreased. Significant elevations of arterial plasma NE, epinephrine, and dopamine occurred after T-2, with metabolic acidosis, hypocarbia, and hyperoxemia in conscious rats and guinea pigs. In pithed rats, T-2 did not increase basal or stimulated plasma catecholamines. The data are consistent with the hypothesis that T-2 toxin disrupts cellular aerobinc metabolism, resulting in lactic acidosis, sympathoadrenomedullary activation, variable initial circulatory responses, and eventual circulatory collapse.

celiac, thoracic, and cervical sympathetic ganglia of cynomolgus and rhesus monkeys. The Quin-2 technique was used to measure intracellular calcium responses to incubation of adrenomedullary cells with CRF, and the effects of CRF on adenylate cyclase activity and catecholamine secretion were measured. Eleven healthy volunteers were given ovine CRF 30 mins after receiving either placebo or morphine sulfate.

Dental patients were assigned randomly to receive or not to receive: intravenous diazepam; epinephrine in the local anesthetic; the opioid, fentanyl; or the opioid antagonist, naloxone. Hemodynamic measures were obtained, as well as blood samples for plasma catecholamines and immunoreactive beta-endorphin.

In conscious and pithed rats and guinea pigs, T-2 (0.5-2 mg/kg iv) was injected and the effects on circulatory and biochemical measures observed. The pithed rat preparation was used to evaluate possible direct effects of T-2 on the heart or vasculature.

The possibility that ANF may increase sodium excretion via renal production of dopamine was examined by observing the effects of a left renal artery injection of 1 mcg/kg ANF followed by an infusion of 0.1 mcg/kg/min over 30 mins in 5 anesthetized dogs. Blood was collected from the left renal artery and vein for measurement of plasma NE and DOPA and urine from the left ureter for measurement of NE and DA.

Results and Their Significance:

The effects of sodium chloride on systolic and diastolic blood pressure of baboons were minimal regardless of the route of administration, but salt loading combined with DOCA rapidly (within days) produced persistent increases in pressure which averaged 20-30 mm Hg. DOCA/salt hypertension was associated with increased water intake, decreased plasma K, and decreased plasma renin activity, but there were no consistent changes in plasma catecholamines or heart rate. A behavioral conditioning procedure, instituted to examine the possibility of ameliorating DOCA/salt hypertension non-pharmacologically, was largely unsuccessful. When clonidine, hydrochlorothiazide with triameterene, and atenolol were tested for their antihypertensive effects during DOCA/salt hypertension, only the diuretic combination affected blood pressure. This is the first report of DOCA/salt hypertension in primates, and the results indicate that in this low renin hypertensive model, increased sympathetic nervous activity does not play an important role.

Six of 9 baboons who underwent diastolic blood pressure conditioning had progressive, significant increases in resting, inter-session averaged pressure (mean increase 8.4 mm Hg diastolic during conditioning among the 9 subjects). Baseline, pre-training heart rate and diastolic pressure predicted the magnitude of the inter-session increase in pressure ($r=0.70$ and $r=0.67$, $p<0.05$ in each case). Two animals had nephrosclerosis and one fibrinoid vascular necrosis. Primates experiencing repeated, conditioned pressor episodes can develop increases in resting blood pressure, but

Administration of ANF increased urinary sodium excretion in 4 of 5 dogs studied. There were no changes in renal blood flow, glomerular filtration rate, or left ventricular diastolic pressure during ANF-induced natriuresis. No change was seen in renal arterial or venous DOPA or NE levels following ANF infusion, and DA excretion was unchanged. The results suggest that the renal production of DA is not involved in the action of ANF in increasing renal excretion of sodium and decreasing renal release of renin.

Proposed Course of Project:

The baboon facility at Johns Hopkins has closed, and so further studies about primate hypertensive models are feasible in the near future. We will continue to collaborate with other groups interested in neuroendocrine systems especially involving CRF, ANF, benzodiazepine receptors, vasopressin, and endogenous opioids. A clinical protocol in collaboration with the NIDR has existed for a few years involving hypertensives undergoing dental surgery, but so few subjects have participated so far that this project may be abandoned this year, and a laboratory form of stress may be substituted.

References:

Turkkan J.S., Harris A.H., Goldstein DS. Do chronically repeated pressor episodes cause sustained elevations in blood pressure? In Weiss SM, Matthews KA, Detre T, Graef JA (Eds), Stress, Reactivity, and Cardiovascular Disease. NIH Publication No. 84-2698, 1984, p. 235.

Dionne R, Goldstein DS, Wirdzek PR, Keiser HR, Dubner R. Effects of diazepam premedication and epinephrine-containing local anesthetic on circulatory and plasma catecholamine responses to minor surgery. Anesthes Analg 63:640-646, 1984.

Feuerstein GZ, Goldstein DS, Ramwell PW, Zerbe RL, Lux WE, Jr, Faden AI, Bayorh MA. Cardiorespiratory, sympathetic and biochemical responses to T-2 toxin in the guinea pig and rat. J Pharmacol Exp Therap 232:786-794, 1985.

Rittmaster RS, Cutler GB Jr, Sorbel DO, Goldstein DS, Koppelman MCS, Loriaux DL, Chrousos GP. Morphine inhibits the pituitary-adrenal response to ovine corticotropin releasing factor in normal subjects. J Clin Endocrinol Metab 60:891-895, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 HL 01975-02 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of renin-angiotensin system in vasoconstriction by acetylcholine in dogs.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Yun	Guest Worker	HE, NHLBI
Others:	J.R. Gill, Jr.	Senior Investigator	HE, NHLBI
	H.R. Keiser	Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of the renin-angiotensin system in the renal vasoconstriction by acetylcholine (ACh) in indomethacin (INDO)-treated dogs was examined in dogs receiving infusion of either saralasin, an angiotensin II blocker (intrarenal), saralasin and phenoxybenzamine (intrarenal) or propranolol (I.V.). In INDO-treated dogs receiving an infusion of saralasin alone or in combination with phenoxybenzamine and propranolol, renal arterial infusion of ACh produced an initial rise in sodium excretion ($U_{Na}V$) and renal plasma flow (RPF) with no change in renin secretion (RS). This was followed by a fall in $U_{Na}V$ and RPF with an increase in RS. It is concluded that ACh produces renal vasoconstriction in INDO-treated dogs by mechanism(s) other than an increase in the activity of the renin-angiotensin system.

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Objectives: Infusion of acetylcholine (ACh) into a renal artery of the dog is known to cause an increase in renal plasma flow (RPF) and sodium excretion ($U_{Na}V$). How ACh produces renal vasodilation and natriuresis is not clear. Recently, we have found that the synthesis of prostaglandins is required for the vasodilatory and natriuretic response to ACh. In that study renal arterial infusion of ACh in control dogs produced a sustained increase in RPF and $U_{Na}V$ without a change in glomerular filtration rate (GFR) or renin secretion rate (RSR). In dogs pretreated with indomethacin (INDO), an inhibitor of PG synthetase, renal arterial infusion of ACh produced an initial increase and then a decline in RPF and $U_{Na}V$ that was accompanied by a progressive fall in GFR, and progressive rise in RSR. The present series of experiments was to determine whether pretreatment of the dog with an angiotensin II blocker, saralasin, or a combination of saralasin, phenoxybenzamine, and propranolol will prevent the renal vasoconstriction by ACh in INDO-treated dogs.

Methods: Mongrel dogs were given a diet containing 180 mEq/day of sodium for one week before study. On the morning of the experiment, the dogs were anesthetized with sodium pentobarbital (30 mg/kg/i.v.) and ventilated with a respirator. Tygon catheters were placed in a femoral artery for the collection of arterial blood samples and measurement of blood pressure and in a femoral and a jugular vein for infusion.

The abdomen was opened through a mid-line incision and tygon catheters were inserted into both ureters for collection of urine and into the left renal vein via the left ovarian vein for the collection of renal venous blood samples. A 20-gauge needle connected to a tygon catheter was inserted into the left renal artery for infusion into the left kidney. A flow probe (Micron RC 1000 was placed on the left renal artery for measurement of renal blood flow (RBF).

One thousand ml of lactated Ringer solution was given during the operative procedures followed by an infusion of lactated Ringer solution at 5.2 ml/min. Inulin 30 mg/kg dissolved in saline was given as primer, followed by a sustaining infusion at 0.58 ml/min to maintain a plasma concentration of approximately 20 mg/100 ml. Urine was collected at 20-minute intervals and, at the mid-point of each collection, arterial (15 ml) and left renal venous (5 ml) blood samples were drawn. When a sample was taken, an equal amount of blood from donor dogs was given as replacement.

After the surgical procedures had been completed, normal saline was infused into the left renal artery at 0.1 ml/min.. When urine flow rate had stabilized, the dogs were then studied according to the following protocols:

Group I: Intrarenal infusion of ACh (7 dogs) - After three consecutive 20-minute control clearance periods were obtained, ACh was then infused into the left renal artery at 40 µg/min for five 20-minute periods. Left renal arterial infusion of saline was then resumed for two 20-minute post-control periods.

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Group II: Intravenous injection of indomethacin plus intrarenal infusion of ACh (10 dogs) - After a 20-minute control clearance period, a bolus of indomethacin (5 mg/kg) was given intravenously and two 20-minute clearance periods were obtained. ACh was then infused into the left renal artery at 40 µg/min for five 20-minute periods. Saline was then infused into the left renal artery for two 20-minute post-control periods.

Group III: Intravenous injection of indomethacin plus intrarenal infusion of saralasin and ACh (4 dogs) - A bolus of indomethacin (5 mg/kg) was given intravenously and thirty minutes later a clearance period was period. An angiotensin II blocker, saralasin was then infused into the left renal artery (76 µg/min). In preliminary experiments, this dose of saralasin has been shown to effectively prevent the vasoconstriction of intrarenal injection of angiotensin II. Saralasin was infused for nine 20-minute periods. After saralasin had been infused for two periods ACh (40 µg/min) was then added to the perfusate for five periods.

Group IV: Intravenous injection of indomethacin and propranolol plus intrarenal infusion of phenoxybenzamine, saralasin and ACh (6 dogs) - The experimental protocol in this series of experiments was the same as that of Group III, except that both saralasin (76 µg/min) and phenoxybenzamine (0.5 mg/kg, followed by 190 µg/min) were infused into the left renal artery. Furthermore, propranolol was infused into a femoral vein (5 mg/kg, followed by 0.66 mg/min).

Significance: Renal arterial infusion of ACh produces a natriuresis and an increase in renal plasma flow without a change in glomerular filtration rate (GFR) or renin secretory rate (RSR). In dogs pretreated with INDO, an inhibitor of prostaglandin synthesis, renal arterial infusion of ACh produced an initial increase followed by a decrease in sodium excretion, RPF, and GFR, and a progressive increase in RSR. The decline in sodium excretion, RPF, and GFR, and the rise in RSR with ACh in INDO-treated dogs could not be prevented by prior treatment with saralasin alone or a combination of saralasin, phenoxybenzamine and propranolol. The present findings suggest that ACh produces renal vasoconstriction in INDO-treated dogs by mechanism(s) other than an increase in the activity of renin-angiotensin system.

Proposed Course of Study: The mechanism(s) by which ACh produces renal vasoconstriction in INDO-treated dogs remains unclear. It is possible that ACh causes an increase in the influx of calcium from the extracellular fluid space into intracellular fluid space in INDO-treated dogs. If this is so, pretreatment with calcium-entry blockers, such as verapamil, would be expected to prevent the vasoconstriction by ACh in INDO-treated dogs. This possibility is currently being examined.

Publications:

1. Ho, S.S., Yun, J.C.H., Gill, Jr., J.R., Kelly, G.D. and Keiser, H.R.: Renin-angiotensin and adrenergic nervous system do not mediate renal vasoconstriction induced by acetylcholine in indomethacin-treated dogs. Renal Physiology (in press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01977-02 HE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Intracellular calcium levels: 1)mechanisms of secretion 2)relation to hypertension.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigetor.) (Name, title, laboratory, and institute affiliation)*

PI:	R. Zimlichman	Visiting Associate	HE, NHLBI
Others:	D. Goldstein	Senior Investigator	HE, NHLBI
	H.R. Keiser	Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

Laboratory of Cell Biology and Genetics, NIADDKD, NIH (H. Pollard)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. We tested the mechanism of secretion in bovine adrenomedullary cells. We found that catecholamine release correlates best with calcium influx and less with free intracellular calcium levels. Furthermore we found conditions in which increases in intracellular calcium levels were not accompanied by release. We conclude that calcium in specific sites within the calcium channel is essential for initiation of the secretory process.

We also found that high K^+ and nicotine activate two different calcium channels which are functionally distinct.

2. We have shown that there is no difference in resting intracellular calcium levels in platelets of SHR and WKY rats when the cells are in the basal state or after various perturbations.

Blockade of Na-K pump with Ouabain does not cause permanent change in intracellular calcium levels. Multiple effective mechanisms buffer quickly any changes in intracellular calcium induced by blockade of the Na-K pump. These results refute Blaustein's hypothesis which suggests that in hypertension intracellular calcium levels are elevated due to a circulating inhibitor of Na-K ATP'ase.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description: ZO1 HL 01977-02 HE

Objectives: Our objectives were to determine: 1. the role of intracellular free calcium in release of catecholamines from adrenomedullary cells, and 2. the role of intracellular free calcium in hypertension including a test of Blaustein's hypothesis that levels of intracellular calcium are elevated in hypertension due to stimulation of a transmembrane sodium-calcium exchange mechanism by a circulating inhibitor of sodium-potassium ATPase.

Methods: Bovine adrenomedullary cells were enzymatically dissociated, kept in primary culture and intracellular free calcium was determined by the Quin 2 method as reported in detail last year. Calcium influx was measured by use of calcium-45 and catecholamines were assayed by high performance liquid chromatography and electrochemical detection. Platelets and thymocytes were obtained from spontaneously hypertensive (SH) and normotensive Wistar Kyoto (WKY) rats. Platelets were separated by gel filtration on Sepharose 2BCL.

Results: 1. Basal levels of intracellular calcium in adrenomedullary cells were 142±6 (n=43) nM, and increased upon stimulation to 318±20 after nicotine, 329±18 after high K^+ and 224±40 nM after Veratridine. Stimulation with nicotine on high K^+ produced a rapid increase in the rate of ^{45}Ca influx. The pattern of changes in intracellular calcium and the rate of return to basal values were different for the different secretagogues. Veratridine caused a relatively small increase in intracellular calcium but the amount of catecholamine released was the largest. A calcium Ionophore increased intracellular calcium but did not affect catecholamine release.

There is no increase in intracellular calcium and no additional catecholamine release upon sequential stimulation with nicotine. However high K^+ after nicotine stimulation produced further increases in intracellular calcium and in catecholamine release. Repetitive stimulation with high K^+ yields repetitive increases in intracellular calcium and in catecholamine release. However high sodium in the medium inhibited the effect of high K^+ but not the effect of nicotine on calcium levels in the cells.

2. No changes in basal free intracellular calcium levels were found in platelets from SHR (115±16 mM), and WKY rats (116±21 nM). Continuous monitoring of free intracellular calcium levels during calcium depletion, repletion and during stimulation with thrombin, ADP and serotonin showed similar maximum responses and rates of rise in intracellular calcium between the two groups of rats.

No changes in free intracellular calcium levels were found in three cell lines, i.e. lymphocytes (121±10 nM), platelets (124±7 nM) and adrenal cells (132±16 nM) during incubations with Ouabain (10^{-3} to 10^{-5} M) for up to 60 minutes. When calcium was added to calcium depleted cells, Ouabain incubation caused faster initial influx of calcium but the levels returned quickly to control levels.

Significance: 1. Our studies prove that the rate of catecholamine release correlates best with the rate of calcium influx and less well with the level of intracellular calcium. We have also shown that in specific conditions increases in intracellular calcium were not accompanied by any catecholamine release. We suggest that the presence of calcium in specific sites within the calcium channel might be the factor which initiates the secretory process. By studying the patterns of response to different stimulants and inactivators we have also shown that nicotine and high K^+ affect two different channels via which calcium enters the cell.

2. We have shown that there is no difference in free intracellular calcium between SHR and WKY rats, under basal conditions or after various perturbations. These data question the suggestion that free intracellular calcium levels are increased in hypertension and that they are etiologically related to the hypertension.

Incubation of three different cell types in Ouabain does not change their level of intracellular calcium. This proves our assumption that the rapid and effective calcium buffering mechanisms prevent changes in free intracellular calcium levels and refute Blaustein's hypothesis.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01986-01 HE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Factors regulating atrial natriuretic peptide release from the atria.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Zamir	Visiting Associate	LCS, NIMH
Others:	H.R. Keiser	Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. The major circulating forms of ANPs in the rat are αR-ANP (5-28) (atriopeptin III) and αR-ANP (3-28) [ANF(8-33)].

2. Concentrations of ANPs in plasma of conscious rats ranged from 80-120 pg per milliliter.

3. Mechanical and hyperosmotic stimuli enhance the release of ANPs in the conscious rat.

4. Halothane anesthesia elevates concentrations of ANPs in plasma of anesthetized rats.

5. Neuronal influences appear to modulate the volume load-induced release of ANPs from rat atria.

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Project Description: ZO1 HL 01986-01 HE

Objectives: 1. Characterization of the atrial natriuretic peptides (ANP) in the plasma of the rat. 2. Elucidation of factors which regulate release of atrial natriuretic peptides from the rat atria.

Methods: We have generated an antiserum against α-rat ANP_{5-28} (ATP III). On the basis of cross-reactivity studies, this antiserum appears to be both conformational and mid-portion directed. It recognizes various ANP'S as follows: α-$RANP_{3-28}$ (ANF_{8-33}), α-$RANP_{4-28}$(Auriculin B), and α-$RANP_{5-28}$(ATP III), α-$RANP_{5-27}$(ATP II), and α-$RANP_{5-25}$(ATP I) on an equimolar basis. In contrast it binds to α-$RANP_{4-24}$(Auriculin A) with a 2.5 fold greater avidity than ATP III and it recognizes α-$RANP_{1-28}$(ANF) and α-human ANP_{1-28} only 10% and <0.2% as well as ATP III, respectively. The characteristics of this antiserum in a routine overnight RIA reveal a maximum sensitivity of 3 PG per tube with an intra-assay sample variation of 3% and an inter-assay sample variation of 5%.

Chronic, indwelling catheters were placed in the femoral vein and artery of adult male rats (Sprague-Dawley, 325-375 gms) 24 hours prior to the experimental manipulation. Catheterized animals received an infusion via the femoral vein of either isotonic saline (20 ml/kg body weight), 5% glucose (20 ml/kg body weight), or hypertonic saline (1 ml containing 2.8 mEq NaCl) over a duration of 1 minute. Since preliminary results revealed that peak circulating levels of IR-ANP occurred between 1 and 2 minutes post infusion, 1.5 ml of blood was withdrawn from the femoral artery at approximately 1.5 minutes and placed immediately in prechilled tubes containing 2.25 mg of EDTA and 1 TIU Aprotinin. Cellular elements were separated by centrifugation at 4°C and plasma was decanted into tubes previously placed in dry ice. Each plasma sample was stored at -60°C until assayed. Prior to the determination of IR-ANP levels, plasma samples were thawed and IR-ANPS were extracted from plasma.

Results: The major circulating forms of ANPs are α-RANP (5-28) (atriopeptin III) and α-RANP (3-28) [ANF(8-33)]. IR-ANP levels in conscious, unrestrained control animals with indwelling catheters were found to be 125±15.6 (mean ± SEM) or 80±10.2 pg/ml in two independent experiments. Our reported basal ANP levels in conscious rats constitute the first report of IR-ANP levels in non-anesthetized, non-traumatized animals and with one exception were found to be 1/5-1/15 of previously reported values. Volume loading (VL) with either 5% glucose or 0.9% saline enhanced plasma levels of IR-ANP 4-5 fold. Hyperosmotic challenge also resulted in a rapid increase in plasma ANP similar to that observed following volume loading.

Clearly, volume expansion or hyperosmotic challenge is a rapid, potent inducer of ANP release; however, whether or not this release is due to a direct effect on the ANP-containing cardiocytes or via an indirect reflex mechanism involving the central nervous system is not known. In an attempt to clarify the role of a neuronal reflex versus a direct cardiac effect as an explanation for observed VL-induced release of ANP, groups of animals

were anesthetized with Halothane, followed by either bilateral vagotomy or sham vagotomy and VL. Exposure to Halothane for 15 minutes resulted in a 3-fold increase in plasma ANPS. In the same experiment, 25 minutes after the onset of anesthesia, animals were vagotimized or sham operated and a third blood sample (PRE VL2) taken at 30 minutes post anesthesia (5 minutes post vagotomy). Animals remained under anesthesisa for an additional 10 minutes at which time each animal was volume loaded with 0.9% naCl and a final blood sample (VL, NaCl) was taken 1.5 minutes later. Vagotomy in Halothane-anesthetized rats did not alter circulating ANP levels or modify VL-induced release of ANPs, therefore PRE VL2 and VL NaCl ANP samples were pooled for statistical analyses from sham and vagotomized animals. Clearly, Halothane anesthesia elevates basal ANP levels throughout the duration of anesthesia, and blocks the VL-induced release of ANPS. Since the site of action of VL-induced release of ANPS is not known, whether or not the blocking action of Halothane occurs directly at the myocardial level or indirectly via alterations of neuronal input to the heart is not clear. In contrast to our observed effects of Halothane anesthesia, pentobarbital anesthesia has been reported to not block VL-induced release of ANPS. Even though changes in circulatory ANP levels induced by VL in pentobarbital-treated animals parallel our observed changes in conscious animals, caution should be exercised in the interpretation of results obtained in anesthetized animals because of the possibility of the differential effects of anesthetics on myocardial and neuronal tissue.

Complete cardiac denervation in the pithed-rat preparation, which removes both humoral influences of central nervous system origin and direct neuronal control of the heart via the vagal and sympathetic nerves, blocked the VL-induced release of ANPS.

Purposed Course of Study: Further experiments are needed to clarify involvement of brain, pituitary gland and adrenal glands in regulation of ANPs release from the atrium.

Publications:

1. Jacobowitz, D.M., Skofitsch, G., Keiser, H., Eskay, R.L. and Zamir, N.: Evidence for the existence of atrial natriuretic peptide containing neurons in the rat brain. Neuroendocrinology 40: 92-94, 1985.

2. Zamir, N., Skofitsch, G., Eskay, R. and Jacobowitz, D.M.: Distribution of immunoreactive atrial natriuretic peptide in the central nervous system of the rat. Brain Research (in press).

3. Skofitsch, G., Jacobowitz, D.M., Eskay, R.L. and Zamir, N.: Immunohistochemical localization of atrial natriuretic peptide in the central nervous system of the rat. Neuroscience (in press).

4. Zamir, N. and Maxiner, W.: The relationship between cardiovascular and pain regulatory systems. Ann. N.Y. Acad. Sci. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01987-01 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effect of Atriopeptin III on systemic hemodynamics.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. Marks	Guest Worker	HE, NHLBI
Others:	Z. Zukowska-Grojec	Guest Researcher	NIB, NINCDS
	T. Ropchak	Biologist	HE, NHLBI
	H.R. Keiser	Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Extracts from mammalian atrial tissue contain peptides, now referred to as atrial natriuretic factor(s), which possess natriuretic, diuretic, and vasorelaxant properties. Three bioactive atrial peptides, the atriopeptins, have been sequenced by Currie et al. (Science 223: 67-69, 1984). Previous reports from other laboratories have been based on bolus injections of either atrial extracts or synthetic ANF peptides and have focused on decreases in blood pressure. Our experiments have utilized a constant infusion of atriopeptin III, the 24-amino acid form. We have found that the fall in mean arterial pressure is due predominately to a decrease in cardiac output. This is secondary to a fall in stroke volume with no change in heart rate and is accompanied by a rise in peripheral resistance and not a fall as others have hypothesized.

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Project Description: ZO1 HL 01987-01 HE

Objectives: Our experiments were designed to evaluate sequentially the effects on systemic hemodynamics of a bolus injection followed by a one hour infusion of rat atriopeptin III (rAP III). The response of the sympathetic nervous system as reflected by plasma levels of norepinephrine, epinephrine, and dopamine was simultaneously evaluated. In addition the effect of rAP III on the cardiovascular response to acute volume expansion was also defined.

Methods: Cardiac output was measured by the thermodilution technique developed and described in detail in annual report project number ZO1 HL 01944-2 HE. Adult rats of the Wistar Kyoto and Spontaneously Hypertensive strains were employed. Rat AP III was infused intravenously at doses of 0.3 and 3 µg/kg/hr following bolus injection of 2 and 20 µg/kg respectively. Sequential hemodynamic measurements were made at five to twenty minute intervals. Acute volume loading with 0.9% saline at 20 ml/kg for one minute was done both alone and during the 3 µg/kg/min rAP III infusion. Plasma catecholamines were measured by the radioenzymatic thin layer chromatographic technique both before and following the atriopeptin and volume loading infusions. The Langendorf isolated heart preparation was used to evaluate rAP III chronotropic effects.

Results: The fall in mean arterial pressure observed with rAP III infusion in both rat strains (-23% in SHR, -21% in WKY) is due predominately to a decrease in cardiac index (-26% and -24%), secondary to a fall in stroke volume (-30% and -23%) and is associated with in increase in peripheral resistance (+14% and +16%). Plasma catecholamines demonstrated no significant change. Heart rate at either dose was not affected by rAP III infusion in vivo or in the Langendorf preparation. The maximal responses in cardiac index, stroke volume, and fall in peripheral resistance induced by acute volume loading were unaffected by the atriopeptin. In addition, although the plasma levels of ANF with the 2 µg/kg bolus dose were comparable with those noted with volume expansion alone, the hemodynamic responses were completely different. Bolus injection decreased both cardiac index (CI) and peripheral resistance (PR) while volume loading increased CI and decreased PR.

Significance: The role of the atrial natriuretic factors in volume homeostasis and their possible therapeutic value in hypertension and situations involving fluid overload is presently under intense study. Experiments performed in the intact animal undergoing a variety of physiologic and pathophysiologic stresses provide fundamental information concerning the mechanism of action of these peptides.

Proposed Course of Study: This study has been completed and the data submitted for publication. New experiments are underway to further investigate the effect of AP III on catecholamine release, baroreceptor function, and sodium balance in renal insufficiency.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZO1 HL 01988-01 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The interaction of rat atrial natriuretic peptide with sympathetic nervous system.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Z. Zukowska-Grojec	Guest Researcher	OD, NINCDS
Others:	M. Haass	Visiting Fellow	NIB, NINCDS
	H.R. Keiser	Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. Alpha rat atrial natriuretic peptide (α-RANP, atriopeptin III), the major circulating form of ANP's, inhibits pressor responses to angiotensin II, vasopressin, norepinephrine (NE) and clonidine when bolus-injected into the pithed rat.
2. A single bolus injection of α-RANP does not alter the acute pressor α-1-adrenoceptor mediated responses to sympathetic stimulation and phenylephrine. Neither does ANP appear to affect plasma NE concentration during stimulation or administration of NE.
3. Acute α-1-mediated responses are also resistant to calcium channel blockers (e.g. nifedipine) whereas α-2-mediated responses to NE or to clonidine, as well as those to angiotensin II and to vasopressin, are dependent on extracellular calcium and blocked by nifedipine.
4. Later phases of all pressor responses become more dependent on calcium flux and are similarly blocked by nifedipine and α-RANP.
5. This pattern of α-2-adrenoceptor specificity during acute pressor responses is not shared by a general vasodilator, sodium nitroprusside, which equally inhibits acute α-1- and α-2-mediated responses.
6. Constant infusion of α-RANP into pithed rat inhibits α-1-mediated pressor responses to sympathetic stimulation and also decreases stimulation-induced NE release.
7. It appears that in vascular tissue the noradrenergic neuroeffector function is relatively unavailable to bolus-injected α-RANP but the presynaptic neuronal release of NE becomes diminished when exposed to longer term infusion of the peptide.
8. Mechanisms of antipressor action of α-RANP appear to involve extracellular calcium flux in both the presynaptic neurone and the vascular smooth muscle.

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Project Description: ZO1 HL 01988-01 HE

Objectives: 1. Assessment of antipressor action of α-RANP in the pithed rat. 2. Presynaptic and postsynaptic effects of α-RANP in noradrenergic neuroeffector functions.

Methods: We used the pithed rat model which enables us to study the cardiovascular system without the interference of centrally mediated circulatory reflexes. Through a pithing rod inserted in the spinal cord, sympathetic nerves can be stimulated at various frequencies evoking predictable pressor responses proportional to log of plasma norepinephrine concentrations. Both presynaptic and postsynaptic mechanisms can be studied. The model has been well described and widely used by us and others.

Plasma norepinephrine and epinephrine concentrations were measured by radioenzymatic, thin-layer chromatographic method as described previously. Rats with bilateral adrenal demedullation were used in the studies in order to avoid confusion with the effects of epinephrine.

Specific α_1- and α_2-agonists (phenylephrine and clonidine) and α_2-antagonists (uphinenine) were used.

Results and Significance: Alpha-rat atrial natriuretic peptide (0.1-10 nmol/kg) was found to cause mild hypotension without affecting heart rate in pithed rats. However, it exerted an antagonistic activity against pressor hormones: angiotensin II, NE and vasopressin (up to 21%, 15% and 33% inhibition).

Angiotensin II-induced pressor responses were most readily blocked by α-RANP suggesting the possibility of an interaction between the two hormones under physiological conditions. The lowest effective doses of α-RANP (0.1-1.0 nmol/kg) yielded concentrations in plasma of immunoactive ANP which were similar to those observed in conscious rats following various volume or osmotic stimuli (100-300 pg/ml). Higher doses of α-RANP (1.0-10 nmol/kg) were required to inhibit vasopressin-induced pressor responses but the resultant percentage of inhibition was the greatest for any hormone studied. Bolus-injected α-RANP (0.1-10 nmol/kg) did not affect acute pressor responses to sympathetic stimulation (SNS, 1Hz, 50V) and α_1-adrenoceptor mediated responses to NE after α_2-blockade. Alpha RANP did not alter the SNS-induced release or overflow of NE into the circulation.

Pressor responses to SNS have been found to be predominantly mediated by α_1-adrenoceptors whereas responses to norepinephrine are mediated mostly by extra-junctional α_2 receptors.

Using the same pithed rat model, VanMell et al. (1981) have also reported that calcium channel blockers selectively inhibit α_2-mediated pressor responses while leaving α_1-mediated responses unaffected. This and other studies have suggested that acute activation of α_2-adrenoceptors requires an influx of extracellular calcium whereas α_1-adrenoceptor stimulation induces mobilization of intracellular calcium. In the present study we explored the specificity of interaction of α-RANP with adrenoceptor subtypes and compared it with calcium channel blocker nifedipine and a general vasodilator, sodium nitroprusside.

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Acute pressor responses to bolus-injected clonidine (α_2-agonist) were dose-dependently attenuated by injection of either α-RANP (up to 21%) or nifedipine (up to 37%) but acute pressor responses to phenylephrine (α_1-agonist) were unaffected. Sodium nitroprusside equally inhibited pressor responses to clonidine (up to 67%) and phenylephrine (up to 66%).

Pressor responses during constant infusions of clonidine and phenylephrine were attenuated similarly by both α-RANP and nifedipine supporting the notion that later pressor responses become dependent on calcium flux regardless of α-adrenoceptor specificity.

In contrast to the inability of bolus injected α-RANP to decrease pressor or plasma NE responses to stimulation, constant infusion of α-RANP at the rate of 1.5 mmol/kg/ min for 30 minutes significantly attenuated the SNS-induced rise in mean blood pressure (by 30%) and NE release (by 25%). Therefore it appears that in vascular tissue the noradrenergic neuroeffector function is relatively inaccessible to bolus-injected α-RANP but is susceptible to modulation when exposed to an increased concentration of α-RANP for a longer time. The decreased presynaptic neuronal release of NE during SNS in the presence of α-RANP may be due to diminished depolarization-induced calcium entry into the synaptic nerve endings. However, confirmation of this hypothesis required further studies and comparisons with calcium channel blockers. Thus mechanisms of antipressor action of α-RANP appear to involve extracellular calcium flux into both the presynaptic neurone and the vascular smooth muscle.

Proposed Course of Study: Further studies of the mechanism by which ANP interacts with the sympathetic nervous system with special attention to effects on calcium entry into synaptic nerve endings.

Publications:

1. Haass, M., Kopin, I.J., Goldstein, D.S. and Zukowska-Grojec, Z.: Differential inhibition of alpha-adrenoceptor-mediated responses by rat atrial natriuretic peptide in the pithed rat. J. Pharmacol. Exp. Ther. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01989-01 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Hormonal responses to salt in normal and essential hypertension.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.R. Gill, Jr.	Senior Investigator	HE, NHLBI
Others	H.G. Gullner	Guest Worker	HE, NHLBI

COOPERATING UNITS *(if any)*
Dept. of Psychiatry, USUHS, Bethesda, MD (C.R. Lake)
Dept. of Pathology, St. Paul-Ramsey Med. Ctr., St. Paul, MN (D.J. Lakatua)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In previous studies of patients with normal renin essential hypertension those that were salt-resistant were shown to have higher urinary dopamine than those that were salt-sensitive. The salt-resistant patients also suppressed their plasma norepinephrine in response to a high-sodium intake whereas the salt-sensitive patients did not. Similar studies performed in normal subjects indicate that when sodium intake was increased from 9 to 249 mEq/d, sodium retention was only slightly less than that in the salt-resistant but significantly less than that in the salt-sensitive patients. Urinary dopamine did not differ from that in the salt-sensitive but was significantly lower than that in the salt-resistant patients. Plasma norepinephrine also did not differ from that in the salt-sensitive but was significantly higher than that in the salt-resistant patients who suppressed plasma norepinephrine in response to high-salt intake. These results suggest that during a high-salt intake supranormal renal dopaminergic activity in the salt-resistant hypertensives prevents supranormal sodium retention; the dopaminergic nervous system may also mediate the supranormal decrease in plasma norepinephrine in these patients. Supranormal sodium retention that does not suppress plasma norepinephrine may mediate, in part, the increase in blood pressure that occurs during a high-sodium intake in the salt-sensitive hypertensives.

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Project Description: ZO1 HL 01989-01 HE

Objectives: In previous studies of salt-sensitive and salt-resistant patients with normal renin essential hypertension during low sodium (9 mEq/d) and high sodium (249 mEq/d) intakes, these two subsets were observed to differ in their cumulative sodium retention, urinary dopamine, and plasma norepinephrine during the high sodium intake. Since it was not clear how the responses of the two subsets of essential hypertension differed from normal in their response to the two sodium intakes, normal subjects were studied on a similar protocol.

Methods: Five normal subjects were admitted to an air-conditioned ward at the Clinical Center and were fed a constant metabolic diet containing 9 mEq/day of sodium for seven days. Sodium intake was then increased to 249 mEq/d for eight days. Plasma norepinephrine was collected in the morning after overnight bedrest on the last day of the low-sodium intake and on days four and eight of the high-sodium intake. All urine was collected throughout the studies and analyzed for sodium, norepinephrine and dopamine. Blood pressure was measured four times a day after the patient had been supine for five minutes or longer.

Results: The results of the measurements of urinary dopamine and plasma norepinephrine during low- and high-sodium intakes and cumulative sodium retention during high-sodium intake are shown in the following table:

	Low Sodium			High Sodium	
	Urinary DA	Plasma NE	Cumulative Na^+ Retention	Urinary DA	Plasma NE
Patients	µg/d	pg/ml	mEq	µg/d	pg/ml
Salt-sensitive	177±19*	209±28	427±36**	184±16	194±15
Salt-resistant	314±29**	180±19	328±44	300±29**	119±9**
Normal	114±11	184±37	256±46	183±26	173±26

DA = Dopamine
NE = Norepinephrine

*p<0.05 vs. normals
**p<0.01 vs. normals

When sodium intake was increased, the normal subjects showed a cumulative sodium retention of 256±46 mEq which was less than in the hypertensives but was only significantly ($p<0.01$) different from that in the salt-sensitive hypertensives. Urinary dopamine was significantly ($p<0.01$) lower than in both subsets of hypertensives during the low-sodium intake and increased significantly during the high-sodium intake to a value that was not different from the salt-sensitive but still significantly ($p<0.01$) lower than the salt-resistant. Plasma norepinephrine was similar in all three groups during the low-sodium intake and during the high-sodium intake decreased significantly ($p<0.01$) only in the salt-resistant patients. The results indicate that the salt-resistant patients that have the highest urinary dopamine have a cumulative sodium retention that approaches normal. An inverse correlation between cumulative sodium retention and urinary dopamine in the hypertensives as a whole suggests that dopamine may be an important natriuretic factor in these patients. An inverse correlation between urinary dopamine and plasma norepinephrine also suggests that the dopaminergic nervous system may mediate the supranormal suppression of plasma norepinephrine in the salt-resistant

patients. The nearly normal cumulative sodium retention and supranormal suppression of plasma norepinephrine could explain the resistance of blood pressure to an increase in sodium intake. The results also suggest that supranormal sodium retention that does not suppress plasma norepinephrine may be responsible for the sensitivity of blood pressure to a high-sodium intake in salt-sensitive hypertension.

Proposed Course of Study: The results suggest the hypothesis that patients with normal renin essential hypertension have an increased renal reabsorption of sodium chloride, which may be genetic. Those that can respond to the sodium retention by an increase in dopaminergic activity may be able to maintain a relatively normal sodium balance and suppress sympathetic activity thereby preventing an increase in blood pressure when salt intake is increased. Those that cannot increase their dopaminergic activity will in turn show a greater dependence of blood pressure on salt intake. This hypothesis will be explored in future studies.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01990-01 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Clinical physiology of hypertension.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D.S. Goldstein	Senior Investigator	HE, NHLBI
Others:	C.J. Folio	Clin. Nurse Tech.	OD, NHLBI
	R. Chadwick	Biomedical Engineer	BEIB, DRS
	B. Chidakel	Electronics Technician	BEIB, DRS
	R. Bonner	Physicist	BEIB, DRS
	M. Maxwell	Biomedical Engineer	BEIB, DRS
	R. Zimlichman	Visiting Associate	HE, NHLBI
	R.O. Cannon III	Medical Staff Fellow	CB, NHLBI
	H.R. Keiser	Chief	HE, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have measured baroreflex-cardiac sensitivity in patients with essential hypertension and the hyperdynamic circulation syndrome. We noted decreased baroreflex sensitivity in the patients, with an inverse correlation between baroreflex sensitivity and plasma norepinephrine. We have used laser-Doppler flowmetry to demonstrate the absence of reflexive skin microvascular responses in patients with regional sympathectomies. Spontaneous oscillations in skin microvascular flow were observed in the sympathectomized human limbs. We tested the validity of impedance cardiography for non-invasive measurement of cardiac output and stroke volume by comparing the impedance technique with invasive procedures. We are developing a Doppler-ultrasound method for continuous measurements of vascular flow and distensibility. We have tested a circulatory model which predicts the changes in the brachial artery pressure waveform during vasoconstriction and vasodilation. Young patients with essential hypertension have defective modulation of the brachial arterial dicrotic wave, which the model explains in terms of increased arterial rigidity and decreased vasodilator responsiveness.

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Objectives:

We have developed several measurement techniques to study the pathophysiology of hypertension.

The impedance cardiographic technique provides beat-to-beat measures of stoke volume, but the meaning of the obtained waveforms has never been completely described. We tested the validity of the technique for measuring cardiac output, by comparing the results with an invasive procedure -- thermodilution -- in terms of absolute values and percent changes in response to several circulatory perturbations.

The arterial baroreflex is the most powerful, rapidly acting circulatory homeostatic control, and baroreflex sensitivity is decreased in hypertension. We have used several techniques to assess baroreflex-cardiac sensitivity, including injections of phenylephrine and nitroglycerine, the Valsalva maneuver, and external neck suction. We determined if sedation with intravenous diazepam affects arterial baroreflex sensitivity.

Laser-Doppler flowmetry provides a continuous measurement of skin microvascular flow. We used laser-Doppler flowmetry in patients who had undergone sympathectomies, in order to assess the completeness of sympathetic denervation and to determine whether spontaneous oscillations of microvascular flow result from fluctuations in sympathetic activity.

We have found that patients with hypertension have defective vertical modulation of the brachial arterial dicrotic wave in response to a vasoconstrictor or vasodilator, and we developed a circulatory model of the arm arterial system to explain this finding.

We currently are developing the Doppler-ultrasound method for separately measuring forearm flow and vascular distensibility. The latter appears to be decreased early in the development of essential hypertension.

Methods:

We compared values for cardiac output using the non-invasive impedance technique with values obtained using an invasive procedure -- mainly thermodilution -- and assessed hemodynamic responses to manipulations which differentially affect heart rate and stroke volume, including cardiac pacing, the Valsalva maneuver, and administration of vasoconstrictors and vasodilators. Baroreflex-cardiac sensitivity was assessed using the R-R interval response per unit change in systolic blood pressure associated with injections of phenylephrine or nitroglycerine, during and after release of the Valsalva maneuver, and during externally applied neck suction. In 18 subjects, after injection of diazepam to a sedative endpoint, baroreflex-cardiac sensitivity was measured again. Blood samples were obtained for plasma catecholamines.

In 3 patients who had undergone regional sympathectomies, laser-Doppler flowmetric measurements were obtained in the sympathectomized

and intact limbs at rest, during the Valsalva maneuver, and during the cold pressor test, and the results were compared with those obtained in 6 healthy volunteers.

The amount of vertical modulation of the brachial dicrotic wave during nitroglycerine-induced vasodilation and phenylephrine-induced vasocontriction was related to the baseline arterial concentration of NE in healthy subjects and in patients with essential hypertension.

Results and Their Significance:

Among 21 patients, absolute values for cardiac output using impedance cardiography agreed with values using the invasive technique ($r=0.86$, $p<0.001$). The impedance method correctly reported the hemodynamic patterns during the Valsalva maneuver, release of the maneuver, cardiac pacing, premature ventricular contractions, hyperthermia, and intravenous administration of vasoconstrictors (ergonovine, phenylephrine) and vasodilators (nitroglycerine, isoproterenol, dipyridamole). The percent change in cardiac output by impedance cardiography was significantly correlated with the percent change by thermodilution ($r=0.87$, $p<0.001$). Impedance cardiography appears in general to measure validly cardiac output and stroke volume in man.

Preliminary results about sedation with diazepam indicate that diazepam causes decreases in venous plasma NE and in the arteriovenous increment in NE, with decreases in stroke volume, but has no effect on baroreflex-cardiac sensitivity. Our previously reported inverse correlation between baroreflex-cardiac sensitivity and venous plasma NE therefore does not appear to have resulted from individual differences in stress responses to the experimental situation.

No microvascular reflexive responses were observed in sympathectomized limbs. Spontaneous oscillations of microvascular flow often were prominent in sympathectomized limbs, indicating that the oscillations do not depend on spontaneous changes in sympathetic microvascular tone.

The amount of vertical modulation of the dicrotic wave decreased with advancing age and with high blood pressure. The circulatory model explained these findings in terms of increasing vascular rigidity and decreasing small vessel vasodilator responsiveness. Neither abnormality alone could explain the obtained results. We noted a significant negative correlation between the arterial level of plasma NE and the amount of modulation of the dicrotic wave after nitroglycerine in subjects 40 years old or younger, suggesting a sympathetic neurogenic component to the vascular abnormalities observed in relatively young patients with essential hypertension.

The Doppler method for measuring blood flow velocity appears to work, but the ultrasound method for measuring changes in vascular caliber requires an experienced observer and immobilization of the subject's arm.

It is unclear at this point whether the ultrasound technique will be applicable in all subjects.

Proposed Course of Project:

The physiological measurements will be used to determine whether patients with essential hypertension have abnormalities of baroreflex-cardiac sensitivity, vascular resistance, or distensibility at rest or during environmental manipulations such as a laboratory stressor, administration of sympathetic or calcium channel blockers, or sedation.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01991-01 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Plasma catecholamines and sympathetic activity in clinical hypertension.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	R. Stull	Chemist	HE, NHLBI
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COOPERATING UNITS *(if any)*

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have used concentrations of plasma catecholamines to indicate activity of the sympathetic nervous system (SNS) in patients with essential hypertension, orthostatic hypotension, the hyperdynamic circulatory state syndrome, or sympathectomies. We developed clinical methods to estimate synaptic cleft concentrations of norepinephrine (NE), the sympathetic neurotransmitter; neuronal NE uptake (Uptake-1); and regional NE removal. We used clonidine suppression testing to identify patients in whom increased SNS activity contributed to high blood pressure. Measurement of arterial and venous NE in sympathectomized and intact human limbs confirmed the validity of plasma NE to indicate SNS activity. Patients with idiopathic orthostatic hypotension had decreased Uptake-1 activity. Elevated plasma catecholamines, clonidine sensitivity, and excessive responsiveness to isoproterenol characterized a patient with hyperdynamic circulation syndrome. The amount of release of endogenous NE during isoproterenol infusions may indicate pre-synaptic beta adrenoceptor responsivensss. Dopamine does not act as a natriuretic hormone.

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Objectives:

Our previous studies indicated that about 1/5 of patients with essential hypertension have high plasma levels of NE. We conducted clonidine suppression testing to determine if the level of NE indicated the contribution of the sympathetic nervous system to high blood pressure.

To validate the use of plasma NE as an index of sympathetic activity in man, we measured arterial and venous NE in the sympathectomized and intact limbs of patients who had undergone regional sympathectomies.

Several removal processes for NE intervene between the synaptic cleft and the general circulation. To take these into account, we developed techniques to estimate the cleft-plasma concentration gradient and regional neuronal removal of NE. By measuring arterial NE during NE infusions and during yohimbine-induced release of endogenous NE in subjects pre-treated with desipramine, we could calculate the cleft NE concentration associated with a 20 mm Hg sympathetically-mediated pressor response. By infusing simultaneously tracer-labelled NE and tracer-labelled isoproterenol, which is not a substrate for neuronal uptake, and sampling arterial and venous blood at the steady-state, regional total and neuronal removal of NE could be quantified.

Applying these techniques to patients with disorders of circulatory control, we determined whether high levels of catecholamines occur in association with excessive heart rate responsiveness to isoproterenol in the hyperdynamic circulatory state syndrome; and whether neuronal norepinephrine removal is decreased in patients with idiopathic orthostatic hypotension.

To detect possible abnormalities of pre-synaptic beta adrenoceptors in hypertension, we are measuring plasma NE responses to isoproterenol.

To determine if dopamine, the precursor of NE, is a natriuretic hormone, we infused dopamine (DA) at physiological or pharmacological doses and measured plasma concentrations of dopamine required for hemodynamic and natriuretic effects.

Methods:

Mean arterial pressure and plasma NE were measured before and 3 hrs after 300 mcg oral clonidine in 44 patients with essential hypertension and 41 normotensive control subjects of similar age.

In 3 patients who had undergone regional sympathectomies, arterial and venous blood samples were obtained from the intact and sympathectomized limbs at rest and during orthostatic stress or the cold pressor test.

Eighteen healthy volunteers underwent NE infusions during one or more of several conditions: no pre-treatment; pre-treatment with 125 mg oral desipramine; intravenous yohimbine administration; or ganglionic blockade

with intravenous trimethaphan. Target pressor responses were 10, 20, and 30 mm Hg increments in mean arterial pressure.

Fourteen healthy volunteers or patients with mild essential hypertension underwent simultaneous infusions of tracer-labelled NE and tracer-labelled isoproterenol, with arterial and antecubital venous blood sampling at the steady-state and at several time points after the infusion. Eight other infusions were administered to subjects pre-treated with desipramine.

Patients with multiple system atrophy or idiopathic orthostatic hypotension underwent similar infusions, with antecubital venous sampling.

In a patient with excessive tachycardic responses to isoproterenol, labile hypertension, and resting tachycardia, arterial and venous plasma catecholamines were measured at rest and during several provocative stimuli, and the above-described infusions of tracer-labelled NE and isoproterenol were administered.

In 10 healthy volunteers, graded infusions of isoproterenol at 3.5, 7, 14, and 35 ng/kg/min were administered for 20 mins each, and blood samples and hemodynamic records were obtained at the end of each infusion period.

DA was infused for 2 hr at 3 fixed doses (0.03 or 0.1, 0.3, and 3 mcg/kg/min) on 3 separate occasions in 6 healthy men, and the hemodynamic and natriuretic responses related to plasma levels.

Results and Their Significance:

Among patients with essential hypertension, the resting level of plasma NE was significantly related to the decrease in mean arterial pressure 3 hrs after a single dose of clonidine ($r=0.62$, $p<0.001$). The magnitude of the depressor response in the patients also was correlated significantly with the decrease in plasma NE after clonidine ($r=0.60$, $p<0.001$). The results suggest that increased sympathetic outflow plays a pathophysiological role in some patients with essential hypertension, and the combination of high baseline NE with an excessive depressor response to clonidine identifies patients with neurogenic hypertension.

During NE infusions in healthy subjects, linear pressor-log NE concentration relationships were observed. At a pressor response of 20 mm Hg, arterial NE averaged 3647 pg/ml. The pressor-log NE relationship was shifted more than 5-fold to the left during combined ganglionic, alpha-2 adrenoceptor, and Uptake-1 blocade (arterial NE at a 20 mm Hg pressor response was reduced to 684 pg/ml). During yohimbine-induced release of endogenous NE in desipramine-pretreated subjects, arterial NE averaged 467 pg/ml at a 20 mm Hg pressor response. Since the concentration of NE in the synaptic clefts must have been between the values for plasma NE during its infusion and during its endogenous release, we estimated that in healthy people, a 20 mm Hg sympathetically-mediated pressor response is associated with about a 560 pg/ml (3 nM) concentration of NE in the average

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neuroeffector junction. These results may now be compared with those of patients with essential hypertension, in order to separate excessive cleft NE concentrations from excessive vascular responsiveness to endogenous NE as determinants of pressor hyper-responsiveness in essential hypertension.

Upon simultaneous injection of tracer-labelled NE and isoproterenol to a steady state, we found that the arm removes about 50% of arterial NE. Pre-treatment with desipramine, which blocks neuronal removal of NE, decreased the proportionate NE removal in the arm to exactly that of isoproterenol, indicating that the difference in removal of NE and isoproterenol could be accounted for entirely by Uptake-1. We estimated that about 15% of infused NE which is removed in the arm is removed by Uptake-1. The results may now be compared with those in patients with essential hypertension, some of whom may have defective Uptake-1 due to a circulating inhibitor of Na/K ATPase.

Whereas in 27 healthy subjects and in the intact limbs of the sympathectomized patients, venous NE exceeded arterial NE, in the sympathectomized limbs arterial NE exceeded venous by about 40%. This was predicted by results of the NE and isoproterenol kinetic studies (see above). The results show that the arteriovenous increment usually seen in plasma NE depends on local release from sympathetic nerve endings. The presence of an arteriovenous decrement in plasma NE establishes sympathetic denervation. In general, the results confirm the validity of plasma levels of NE to indicate sympathetically-mediated NE release.

The patient with the hyperdynamic circulatory state syndrome had decreased arterial baroreflex-cardiac sensitivity, increased circulating levels of NE and epinephrine, resting tachycardia and increased cardiac index, anxiety, and exaggerated heart rate responses to intravenous isoproterenol. He had a large depressor response to clonidine and no abnormality of NE clearance. Intravenous isoproterenol or yohimbine induced episodes of hypertension and tachycardia which were reversed by intravenous propranolol. Sedation with intravenous diazepam increased the patient's baroreflex sensitivity and decreased his arterial and venous levels of NE. We concluded that the patient had simultaneous parasympathetic inhibition and sympathetic stimulation associated with his hypertension and tachycardia, consistent with a functional derangement of baroreceptor pathways. The hyperdynamic circulatory state syndrome, of which this patient seems a classic example, appears to be associated with neurogenic hypertension.

Intravenous administration of isoproterenol produced dose-related increments in heart rate and cardiac output, with little effect on mean arterial pressure and with decreased total peripheral resistance as indicated by impedance cardiography. Plasma NE increased in a dose-related manner in all the subjects. Plasma levels of epinephrine were unaffected. The results are consistent with the hypothesis that stimulation of pre-synaptic beta adrenoceptors causes release of endogenous NE from sympathetic nerve endings but does not affect adrenomedullary secretion of epinephrine. The results also suggest that epinephrine is not co-released

from sympathetic nerve endings and that isoproterenol can indirectly enhance alpha-1 adrenoceptor-mediated vasoconstriction by stimulating release of endogenous NE. The technique may now be applied to patients with essential hypertension, in order to detect a possible abnormality of pre-synaptic beta adrenoceptor responsiveness.

DA infusions resulted in steady-state plasma DA concentrations of 0.69, 3.7, and 38 ng/ml. Mean DA excretion increased significantly from basal levels during all three DA infusions. NE excretion increased during both the medium and high dose infusions, whereas sodium excretion, plasma NE, and heart rate increased only during the high dose DA infusion. Basal aldosterone values were low and did not change. Since a natriuretic response occurred only at supraphysiological concentrations of circulating DA, if DA has a physiological role it must be released from dopaminergic neurons or otherwise produced locally in very high concentrations in the kidney.

Proposed Course of Project:

We will apply to patients with essential hypertension the techniques for estimating synaptic cleft NE, regional neuronal removal of NE, and pre-synaptic beta adrenoceptor responsiveness. The techniques also may be used to measure regional sympathetic activity in the canine renal or cardiac vascular beds. The relationship between salt-sensitivity and sympathetic nervous system activity will be assessed using clonidine suppression testing during outpatient sodium restriction and loading. We will measure the effects of physical (orthostasis) and psychological (computer game) stresses on NE kinetics and conduct psychological profiling of patients with high or low baseline NE. In anesthetized dogs we will determine the pre- and post-ganglionic origin of cerebrospinal fluid NE using ganglionic blockade or alpha-2 agonism or antagonism.

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5.30

ANNUAL REPORT
SECTION ON BIOCHEMICAL PHARMACOLOGY
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

The Section on Biochemical Pharmacology has directed its major research interests toward the understanding of several neurochemical systems which utilize biogenic amines or neuropeptides as neurotransmitters. Control of biosynthesis, mechanisms of release, and receptor responses to neurotransmitters have been investigated. The role of these systems in blood pressure control and neurological diseases has also been a focus of the research.

I) Enzymic Mechanisms.

A. Tyrosine hydroxylase. Studies on the characterization and regulation of this important enzyme have been a continuing project in our lab for a number of years. Prior studies reveal that the enzyme is a substrate for protein kinases and that it is activated by phosphorylation. This activation revolves around the affinity of the enzyme for the electron donor, tetrahydrobiopterin (BH_4).

Tyrosine hydroxylase (TH) has been purified to homogeneity from cultured PC12 cells. The enzyme has a subunit molecular weight of 58,800. Two dimensional electrophoresis of the purified enzyme reveal three isozymic forms differing in charge (pI between 5.3-5.6) and which were positively identified by Western immunoblots. Amino acid analysis indicated a high content of hydrophobic amino acids which was confirmed by the observation that the enzyme also binds tightly to phenylsepharose, a hydrophobic matrix. Antibodies against PC13 TH were raised in rabbits and the antibody was highly specific. Blots of crude protein preparations from brain, adrenal chromaffin cells, rat striatum, and cultured PC12 cells onto nitrocellulose paper revealed that the antibody recognized a single protein of Mr 58,800 in each tissue which corresponds to the Mr of purified TH. The anti-TH also immunoprecipitated TH enzyme activity from each tissue but interaction of the antibody with TH neither activated nor inhibited enzyme activity as such. The anti-TH does not cross react with other monooxygenase enzymes including tryptophan hydroxylase and phenylalanine hydroxylase. The purified enzyme is a good substrate for various protein kinase enzymes. The enzyme shows little activity toward tryptophan as a substrate and expressed no phenylalanine hydroxylase activity whatsoever.

The in vivo regulation of tyrosine hydroxylase was also investigated. A new compound known as BHT 920 has recently been reported to be a specific dopamine autoreceptor agonist. Since the phosphorylation state of tyrosine hydroxylase in vivo appears to be controlled by presynaptic receptors, it was of interest to determine what effect BHT 920 would have upon the kinetic state of tyrosine hydroxylase. Treatment of rats with this compound resulted in a significant increase in the Km value of striatal tyrosine hydroxylase for BH_4. This kinetic analysis indicated that activation of autoreceptors on dopaminergic terminals in the striatum caused a decrease in the phosphorylation state of the enzyme. The significance of these findings is that in certain disease states which are postulated to result from an overactivity of the dopaminergic system treatment with BHT 920 might be an effective way of controlling dopaminergic hyperactivity.

B. Protein-O-carboxymethyltransferase. Protein-O-carboxymethyltransferase (PCM) catalyzes the transfer of a methyl group from S-adenosylmethionine to aspartyl residues on a variety of proteins. The function of this enzyme is not known, although it has been postulated to participate in the repair of defective proteins. It has also been suggested that it may participate in regulatory events for other enzymes. Previous work in our laboratory had shown that calmodulin binding proteins are particularly good substrates for PCM and that the calmodulin dependent activity of phosphodiesterase and the calmodulin dependent activity of protein kinase are indeed modulated by a protein carboxymethylation. During the past year we examined the possibility of whether the protein phosphatase known as calcineurin could also be regulated by PCM. Incubation of pure calcineurin with pure PCM and S-adenosylmethionine resulted in the incorporation of up to 2 moles of methyl groups per mole of calcineurin. The methyl groups incorporated into calcineurin were rather labile and underwent a rapid spontaneous hydrolysis at pH values above 6.5. Subunit analysis of calcineurin indicated that only the 61,000 molecular weight subunit served as a methyl acceptor. Following methylation, the basal phosphatase actitity of calcineurin was little effected, however, the ability of calmodulin to stimulate the activity of calcineurin was completely blocked. While knowledge of the regulation of protein phosphatase activity is an extremely important subject, we have no indication that PCM performs a regulatory role for calcineurin in vivo.

II. Receptor Mechanisms.

A. Tachykinin receptors. Substance P and related neuropeptides appear to bind to a variety of receptor types in mammalian tissue. Two major types of receptors, the P-type and E-type, have been known for some time. The recent discovery of a related neuropeptide, substance K, prompted us to reexamine the receptor types for the various tachykinins. Using classical ligand binding techniques, a new type of tachykinin receptor was discovered in mammalian tissues. This type shows relative specificity for substance K and has a unique distribution in mammalian tissues. All three receptor sites are regulated similarly by ions and guanine nucleotides. The unique distribution of these receptors indicates that the various endogenous tachykinins perform very specific roles. In other studies, the development of the substance P type receptor in brain was examined during embryonic development and during the first several weeks of life in rats. It appears that the substance P receptors develop coordinately with the development with substance P in the tissue.

B. Adrenergic and neuropeptide receptors in vascular smooth muscle cells. Vascular smooth muscle cells appear to have receptors for adrenergic agents as well as several neuropeptides. The response of these cells to stimulation of these receptors was examined in detail. Stimulation of beta receptors on these cells results in a rapid 600-fold increase in cAMP content. Conversely, beta stimulation has little effect on intracellular-free calcium. In contrast, angiotensin II and arginine vasopressin cause rapid and marked increases in intracellular-free calcium but have little effect on the accumulation of cAMP. In the case of arginine vasopressin, the enhancement of intracellular calcium was dependent entirely upon extracellular calcium. In contrast, angiotensin II appeared to stimulate both uptake of extracellular calcium and release of intracellular calcium. An examination of phosphatidylinositol turnover revealed that this biochemical event was closely correlated with the accumulation of intracellular calcium. Both events were blocked by the appropriate agonist. In

a series of related studies, we have recently discovered that although angiotensin II has little effect on the accumulation of cAMP, it strongly potentiates to normally weak accumulation of cAMP in response to vasoactive intestinal polypeptide. Such a powerful synergism may offer an opportunity for further understanding of how neuropeptides interact at their cellular receptor sites.

A comparison of these receptor responses was evaluated in cells isolated either from the spontaneously hypertensive rats or the normotensive Wistar control rats. No significant differences in the responses of cells from either of these strains of rats was seen with regard to beta stimulation or angiotensin II stimulation. However, the response to arginine vasopressin was significantly enhanced in the cells obtained from the genetically hypertensive animals. The hyperresponsivity of cells from the spontaneously hypertensive rats suggest that arginine vasopressin could possible be one of the participants in the development of hypertension in this genetic model.

C. Somatostatin receptors in brain. The biologically active peptide, somatostatin, has been localized in several hypothalamic and extrahypothalamic brain regions where it may function as a classical neurotransmitter or as a modulator of neural activity. We have studied somatostatin binding sites in rat brain by incubation of tissue sections with ^{125}I-tyr^{1}-somatostatin, Ultrofilm autoradiography, computerized microdensitometry and comparison with ^{125}I standards. Highest concentrations of somatostatin binding sites (fmol/mg protein) were found in the claustrum, central nucleus of the amygdala, deep layers of cerebral cortex, lateral olfactory nuclei, hippocampus, medial and lateral septal nuclei, and the medial habenula. Regulation of somatostatin binding sites may be studied as one approach to examining the involvement of brain somatostatin pathways in various physiological and behavioral states.

III. Uptake and Release of Neurotransmitters.

A. Norepinephrine release in the heart. Noradrenergic neurons play an important role in regulating the activity of the heart. One of the continuing studies in our laboratory has been related to the mechanisms by which norepinephrine is released from nerve terminals in heart. Prior studies have lead to the hypothesis that one of the important mechanisms for norepinephrine secretion was through an active outward transport mechanism rather than a exocytotic release. Electron microscopic studies provided evidence that the vesicular membranes were able to merge with the plasma membrane and that the resulting structure appeared to have a calcium dependent biogenic amine transport system.

B. In vivo release of central serotonin. Utilizing a set of behavioral responses indicative of central serotonin release, it has been possible to investigate the release of serotonin *in situ*. In rats in which the vesicular pool of serotonin had been destroyed by treatment with reserpine, it was possible to elicit the typical serotonin release response following treatment with parachloroamphetamine. This and a series of related pharmacological manipulations suggested that serotonin could be released from cytoplasmic pools and that perhaps this source was one of the major mechanisms of serotonin release in the CNS. These findings are indeed quite consistent with the observations reported above with regard to the release of norepinephrine from cardiac noradrenergic nerves. In a related study, the release of serotonin from synaptosomes was also examined. An HPLC assay with fluorometric detection has been developed which is

sensitive enough to simultaneously measure endogenous tryptophn, serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) inside of synaptosomes as well as that which has been released into the incubation medium. Using this assay, we have observed that tryptophan is rapidly taken up by synaptosomes and turned over to 5-HIAA without a concurrent release of 5-HT. Exogenous 5-HT is also rapidly taken up and, within 20-30 min, 80% of the 5-HT is deaminated. Veratridine releases both tryptophan and 5-HT from synaptosomes. Changes in the disposition of exogenous tryptophan or 5-HT can be completely accounted for by uptake or by stoichiometric changes in metabolites. This assay method should be valuable in the study of 5-HT pools and in the determination of from which pool 5-HT release occurs.

C. Dopamine uptake. Two aspects of dopamine uptake have been studied. In one series of experiments the possible role of dopamine in the neurotoxicity of certain serotonin neurons was evaluated. It is known that parachloroamphetamine and metamphetamine both result in the loss of central serotonin neurons. From our current studies it can be concluded that amphetamine is perhaps working through the release of dopamine and that it is the uptake of dopamine into serotonin neurons and its resulting oxidation that causes the neurotoxicity. The oxidation products of dopamine which are responsible have yet to be identified.

D. Adrenal catecholamine and enkephalin release. Canine adrenal glands served as a model to study neurotransmitter release elicited by GABA receptor stimulation. Infusion of GABA or GABA-mimetics in the adrenal gland increased the release of catecholamines and met-enkephalin-like peptides. This release was a consequence of direct depolarization of chromaffin cell membranes and was not mediated transsynaptically through acetylcholine or met-enkephalin.

The uptake of dopamine by dopaminergic neurons appears to be regulated in part by a receptor system which recognizes cocaine as its ligand. It would appear that specifically in the corpus striatum a sodium dependent cocaine binding site can be thought of as a biochemical marker for dopamine uptake sites. More recently a GABA-modulin like polypeptide has been purified from striatal synaptosomes. This small protein is effective in inhibiting sodium-dependent cocaine binding.

IV. Anatomical Studies.

A. Protein-O-carboxylmethyltransferase. Both biochemical and immunohistochemical techniques were used to demonstrate the presence of protein-O-carboxylmethyltransferase (PCM; E.C. 2.1.1.24) in the CNS. The highest levels of immunoreactivity were detected in cortex, hippocampus, corpus striatum, thalamus, and the amygdala. Other brain areas exhibited lower amounts of immunoreactive PCM. Most of the immunoreactive cells were neuronal with prominent labelling detected in both the cell body and the axonal region. Biochemical analysis of PCM activity correlated with the immunohistochemical localization. Methyl acceptor substrates for the enzyme were also high in regions rich in PCM. Western immunoblot analysis of bovine brain, rat brain and human erythrocyte forms of the enzyme showed the antisera generated against bovine brain PCM cross-reacted with human and rat forms of the enzyme, suggesting structural homology. These results suggest that PCM has an unique neuronal pattern of distribution, and that carboxylmethylation of proteins may be of functional significance in the nervous system. The levels of PCM enzyme activity and

immunoreactivity were sufficiently high in the locus coeruleus (LC) and substantia nigra (SN) to warrant comparison with tyrosine hydroxylase (TH) immunoreactivity. Western blot analysis confirmed that the antibodies were specific and did not cross react with the other antigen of the comparison. Both anti-PCM and anti-TH heavily labelled cells within the LC and SN and it appears that the antigens are colocalized within the same cells in these brain areas.

B. Serotonin pathways in the brain. The origin of the serotonergic (5-HT) innervation of the nucleus tractus solitarius (NTS) was studied by injecting fluorescent dyes or enzymes which are transported in a retrograde direction into the NTS. The dorsal raphe nucleus was also ablated with electrolytic lesions and the effects on 5-HT levels in the NTS and other brain areas were determined by HPLC. Unilateral removal of the nodose ganglion was tested for its effects on NTS 5-HT levels. The results from these studies leave open the question of 5-HT innervation of the NTS since neither raphe lesions nor nodose ganglionectomy altered NTS 5-HT levels or its metabolism in any detectable fashion. Furthermore, fluorescent retrograde dyes could not be reliable traced from the NTS to any major 5-HT nuclei.

V. Protein Phosphorylations.

It appears that synaptic mechanisms are controlled in part by numerous protein phosphorylations. Several protein kinases are present in large amounts in brain tissue and these are regulated either by cAMP, calcium, or phospholipids. It is thought that the activation of receptors on adjacent cells in the CNS is accompanied by changes in the phosphorylation state of a number of proteins. In work during the past year, we have attempted to examine the nature of these phosphate acceptor proteins in synaptosomes. In an effort to work with synaptosomes enriched for a particular type of neurotransmitter, methodology was developed to selectively enrich synaptosomes for either serotonin or dopamine. When the distribution for phosphoproteins in these two populations of synaptosomes was examined, the majority of the phosphoproteins showed no selective enrichment. There were, however, several proteins that were selectively localized in one population or the other. It was of interest to find that one phosphoprotein, tyrosine hydroxylase, was found relatively selectively in the dopamine-rich synaptosomes while another phosphoprotein, DARP 32 was enriched in the serotonergic proteins. The ability to enrich synaptosomal preparations for a particular neurotransmitter will be very important for future studies.

The molecular components of neuronal calcium channels have not yet been identified, however, protein phosphorylation has been shown to play a role in the function of calcium channels. Two neuronal phosphoproteins, P96 and P139, were studied in rat brain synaptosomes. Activation of calcium channels by depolarization leads to the extremely rapid dephosphorylation of these proteins. Although dephosphorylation was dependent upon external calcium, it was also dependent on the mechanism of calcium entry through calcium channels. Thus, agents which stimulated rises in intracellular calcium did not necessarily initiate dephosphorylation. In contrast, drugs which activate or inhibit calcium channels specifically increased the phosphorylation of P96 and P139. The data support a proposal that these phosphoproteins may be involved in calcium channel function.

VI. Blood Pressure Control

A. Role of the serotonin system. There is compelling evidence that central serotonin neurons are involved in the regulation of the cardiovascular system. However, the brain circuitry which underlies this influence is poorly understood. Prior work from our laboratory has shown that stimulation of serotonin neurons in certain of the raphe nuclei result in significant increases in blood pressure suggesting that serotonin neurons in the midbrain are associated with a pressor system. In contrast to this conclusion, it was also reported that the administration of the precursor serotonin, L-tryptophan, causes a reduction in blood pressure. To examine this apparent conflict, studies were done with the administration of either L- or D-tryptophan to both hypertensive and normotensive rats. It was of interest to find that only L-tryptophan caused a reduction in blood pressure while both D- and L-tryptophan caused a significant increase in brain serotonin. Thus, it would appear that the changes seen in the blood pressure of hypertensive rats following tryptophan administration is due to a response not related to central serotonin formation. One conclusion is that it is likely that peripheral metabolites other than serotonin may be responsible for the hypotensive effect of L-tryptophan.

In a separate study, the possible role of the serotonin system in the baroreflex was examined in conscious rats. In this work, serotonergic neurons in specific brain areas were destroyed selectively with the neurotoxin 5,7-dihydroxytryptamine. Following the development of a new system to evaluate the baroreflex in rats, the baroreceptor function was measured. A preliminary evaluation of our findings suggests that these serotonin ligands have little effect or role in the baroreceptor reflex.

B) Nutrition and cardiovascular disease. Prior work from our laboratory had suggested that the protein content of the diet was an important determinant in the incidence of stroke in stroke-prone spontaneously hypertensive rats. Rats that were maintained on a relatively low protein diet had a much higher incidence of cerebral lesions. In an effort to learn about the molecular mechanisms responsible for this protective effect of protein, we examined the effect of long-term treatment with either high or low protein diets on the ability of sarcoplasmic reticulum to take up calcium and on the level of calcium-dependent ATPase. It was found that animals on high protein diets (32%) or methionine supplemented diets had greater calcium-dependent ATPase activity and had a more rapid uptake of calcium into the sarcoplasmic reticulum. These findings are consistent with the concept that diet can play a role in the maintenance of blood pressure and that this role may be exerted by modifying the ability of specific tissues to sequester calcium.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01843-12 HE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanisms of Uptake and Release of Norepinephrine in Adrenergic Nerve Endings

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Donald F. Bogdanski Pharmacologist HE NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	0.5	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Accumulating evidence continues to be supportive of our hypothesis that Ch+-Ca++-stimulated neurosecretion by adrenergic nerve endings is mediated by outward transport of NE in vesicles fused or attached to the plasmalemma. The overall plan is and has been to induce Ca++ dependent neurosecretion by rat heart ventricle slices incubated in a Na+ deprived Krebs bicarbonate medium containing choline Cl as the replacement for NaCl. The integrity of the plasmalemma was shown by a Ca++-dependent, secretion induced by K+ after secretion was slowed by appropriate modification of experimental conditions. Biochemical evidence of vesicle fusion was the demonstration of reactions known to characterize vesicle membranes. Inhibition of secretion by the impermeable ATP is such a reaction. Depending upon the concentration, chlorpromazine, a drug known to have a variety of effects on biological membranes, and to inhibit H+ transport, may increase or decrease Ch+-Ca++ stimulated neurosecretion. Yohimbine had little effect in specific alpha receptor blocking concentrations. Established secretion was reversible by the ommission of extracellular Ca++.

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Objectives: As in previous years, we are studying a Ca^{++}-dependent, model neurosecretory system in peripheral adrenergic nerve endings in rat heart ventricle slices incubated in a modified Krebs bicarbonate medium in which choline Cl replaces NaCl (Ch^{+}-Ca^{++}). Our current objectives are to establish that this neurosecretion, apparently mediated by a cocaine sensitive outward transport, originates from vesicles attached or fused to the plasmalemma.

The approach used to study this problem has been to establish the integrity of the plasmalemma and to establish criteria in support of the hypothesis that vesicle membranes fused to the plasmalemma will be accessible to exogenous substances in the incubation medium. These substances are not known to readily permeate the intact plasmalemma.

In review of previous findings in this laboratory, the intactness of the system as a whole (plasmalemma and vesicles) after prolonged (4 h) incubation of tissues in a Krebs bicarbonate medium (KRB) was shown by the similar T/2 of tritiated norepinephrine in vivo and in vitro. Membrane integrity was also suggested by the relatively slow rate of depletion of tissue ^{3}H-NE during the incubation of tissues in Ca^{++}-free choline^{+} or choline-Ca^{++} media containing cocaine or desipramine. More directly, depletion of tissue ^{3}H-NE by reserpine with the recovery of ^{3}H-deaminated metabolites in the medium establishes the integrity of vesicles. The retention of tissue ^{3}H-NE, and Ca^{++} dependent depletion stimulated by depolarizing concentrations of K^{+} establishes the integrity of the plasmalemma. The inhibition of Ch^{+}-Ca^{++} stimulated neurosecretion when exogenous ATP is included in the medium suggests that vesicles are attached to the axolemma to form a modified morphological unit accessible to specific impermeable substances in the extracellular fluid.

Methods: The methods used to induce the Ch^{+}-Ca^{++} stimulated neurosecretion of ^{3}H-NE in adrenergic nerve endings were previously described in detail and are published. Briefly, rats were injected (i.v.) with ^{3}H-NE and heart ventricle slices were prepared 18 to 24 hours afterwards. After a 90 min preincubation to wash out non-neuronal ^{3}H-NE groups of slices were transferred to beakers containing 20 ml of various media for incubation. Some groups were incubated in Ch^{+}-Ca^{++}. Rapid neurosecretion of ^{3}H-amines began 60 to 80 min after the start of incubation. Substances used to modify secretion were either incorporated in the preincubation and incubation media or in the medium after 80 min of incubation. At this time secretion was maximal. Media were changed by transferring slices from one beaker to another containing the new medium. After varying intervals of incubation, aliquots of incubation medium were transferred to vials for liquid scintillation counting of radioactivity.

Major Findings: Before describing our findings for the year, it should be pointed out that the prolonged absence of this investigator due to back problems requiring surgery made it impossible for him to begin certain projects.

In accord with the previous findings, it was decided to tie up a few loose ends regarding the integrity of the plasma membrane of nerve endings after their prolonged incubation in Ch^{+}-Ca^{++}. The existence of an electrically polarized membrane is taken as proof of an intact axolemma which maintains at least some metabolic activity. Evidence for such a membrane in a peripheral nerve ending is a K^{+} induced, Ca^{++} dependent secretion of neurotransmitter. In this

connection, a contribution of this years work was the establishment of a Ca^{++}-dependency for the secretion induced by K^+ in tissues being incubated in Ch^+-Ca^{++}. Potassium ion (66 mM) stimulated the already rapid secretion. The effect of K was made more obvious after the rate of Ch^+-Ca^{++} stimulated neurosecretion was slowed, either by a slight reduction of temperature to 34° or by the addition of Na^+ (70 mM NaCl) to the standard Ch^+-Ca^{++} medium after 80 min of incubation, when rapid Ch^+-Ca^{++} stimulated secretion had begun. The ommission of Ca^{++} from the medium prevented K^+-induced neurosecretion. Moreover, K^+, in depolarizing concentrations, did not induce rapid neurosecretion in slices deprived of Ca^{++} throughout their incubation. In such slices, rapid neurosecretion did not occur, with or without K^+.

Potassium ion also induced neurosecretion in Ch^+-Ca^{++} stimulated tissues made hypertonic with choline, or sucrose, 63 and 130 mM, respectively, rather than NaCl. These results indicate that the effect of K^+ was not contingent upon the presence of Na^+. Moreover, hypertonicity as such did not accelerate Ch^+-Ca^{++} stimulated neurosecretion. The lack of a K^+-induced neurosecretion in tissues incubated in a Ca^{++}-deprived choline medium supplemented with 130 mM sucrose also showed that the effects of K^+ were Ca^{++} dependent.

It is concluded that K^+ depolarized Ch^+-Ca^{++} stimulated nerve endings. Thus, the axolemmae of these endings appear to be electrically polarized and metabolically functional even after 140 min incubation in the Ch^+-Ca^{++} medium.

Although Ch^+-Ca^{++}-stimulated neurosecretion is reversible by the addition of Na^+, some differences between the neurosecretory responses to Ch^+-Ca^{++} and other Na^+-deprived media are now known. A few differences have been reported by us previously. Another difference involves the effect of an addition of Ca^{++} to a Ca^{++}-deprived choline medium in which tissues were incubated for a prolonged period of time (80 min). Secretion was stimulated to an expected Ch^+-Ca^{++}-stimulated rate without additional delay after Ca^{++} was added. By contrast, other workers have reported marked potentiation of secretion in other tissues after Ca^{++} was added to a Ca^{++}-free sucrose medium in which the tissues were incubated. This is regarded as a result of an abnormally high permeation of the cell membrane by Ca^{++}. After a 20 min exposure of tissues to Ca^{++} in our experiments, the ion was again omitted from a fresh Ch^+-Ca^{++} to which the tissues were transferred. Secretion gradually subsided during the following 40 min of incubation. Thus, secretion is dependent upon the presence of extracellular Ca^{++} indicating that tissue Ca^{++} was effectively bound by normal mechanisms. If released, this Ca^{++} cannot induce secretion. However, the effects of exogenous Ca^{++} outlast its availability in the medium. Either the Ca^{++} was incompletely bound after entry into the tissue or the secretory process once started in individual vesicles goes to completion without Ca^{++}. Secretion stops because other vesicles will not bind to the axolemma in the absence of Ca^{++}.

Metabolic Factors for the Retention of ^{3}H-NE. Chlorpromazine was used in continuing studies on the effects of metabolic factors in the retention and release of transmitter amine. This drug exerts several effects on biologic membranes and thus may exert effects on fused plasma and vesicle membranes.

In high concentrations up to 46 μM chlorpromazine increased the rates of depletion of ^{3}H-NE in tissues incubated not only in Ch^+-Ca^{++} but in KRB as

well. The effects occurred during the first 70 min of incubation in Ch^{+}-Ca^{++} which preceeds the usual onset of rapid secretion. Subsequently, secretion was slowed by chlorpromazine.

In concentrations of 4.6 μM or less, chlorpromazine inhibited the rapid rates of secretion, but did not stimulate secretion during the early part of incubation.

Chlorpromazine in low concentrations uncouples hydrogen transport across membranes, and, if it could be demonstrated to induce neurosecretory responses, this mechanism would be of interest to us. Its stimulatory effects on amine release differ, however, from those of 2,4-dinitrophenol (2,4-DNP) a classic uncoupler of oxidative phosphorylation known to short circut H^{+} transport. As reported last year, 2,4-DNP blocked the inhibitory effect of ATP on Ch^{+}-Ca^{++} stimulated neurosecretion, but it showed little effect in slices incubated in KRB. While chlorpromazine may release Ca^{++} from binding sites on the membranes and thus release NE regardless of the incubation medium, effects which more specifically support the concept of fused membranes are of more interest to us.

Lithium (20 and 40 mM) and DCCD inhibit Mg^{++}-ATPase and both agents block the inhibitory effects of ATP on Ch^{+}-Ca^{++} stimulated neurosecretion (reported last year). In concentrations effective against ATP, Li^{+} showed little effect upon neurosecretion in tissues incubated in KRB, whereas DCCD exhibited a moderate degree of specificity. It was concluded that both Li^{+} and DCCD act upon vesicles which are in the act of secreting neurotransmitter. These effects are evidence for the existence of fused membranes.

Effects of Yohimbine. This drug is regarded as a presynaptic α_2 receptor antagonist which increases the neurosecretion of NE by blocking negative feedback induced by the secretion of α_2 receptor agonist (transmitter). Efforts to increase the rate of Ch^{+}-Ca^{++} stimulated neurosecretion with yohimbine administered in a range of concentrations known to specifically act upon α_2 receptors were negative. A high concentration of yohimbine increased the rate of secretion during the first 70 min of incubation before rapid Ch^{+}-Ca^{++} induced secretion had begun.

Yohimbine and chlorpromazine, as reported for this year and Li^{+} (70 mM) as reported for a previous year, all facilitated the efflux that occurs during the first hour of the incubation of the tissues in Ch^{+}-Ca^{++}. The effect of Li^{+} has been explained as being a response to the inhibition of uptake by vesicles fused to the plasma membrane. In similar concentrations, Li^{+} had little effect on tissues incubated in KRB. In high concentrations, chlorpromazine and yohimbine both released the ^{3}H-NE in tissues which were incubated in KRB so their effects are not regarded as being specific for the vesicle-axolemma secreting unit in Ch^{+}-Ca^{++} stimulated nerve endings. These effects are presently of secondary importance to us. A manuscript describing the effects of Li^{+} has been submitted for publication.

Electron micrographic studies of Ch^{+}-Ca^{++} stimulated nerve endings were begun previously and results were described in the report for last year. We continue to seek examples of vesicle and plasmalemma fused with the elimination of layers. A possible example of such fused membranes was photographed. The

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problem, of course, is to accidently transect the tissue at the correct spot in the nerve ending at the correct angle to view an almost infinitesimal structure.

Significance to Biomedical Research and Institute Programs: As in previous years, we are accumulating evidence which strongly suggests that the neurosecretion of NE by adrenergic nerve endings is mediated by outward transport. This model system can (1) represent normal transmission of adrenergic impulses in peripheral adrenergic nerve endings which show no morphological synapse, (2) represent an intermediate stage in synaptic transmission, or (3) represent random neurosecretory events which can be uncontrolled in pathological states. An integral part of this system is visualized as the vesicle-axolemma functional unit which can be fused, attached, or simply adjacent membranes functionally united by proximity. The function is to secrete transmitter. The membranes are activated by the entry of extracellular Ca^{++} into the nerve ending. The fused vesicle membrane becomes accessible to substances in the extracellular fluid. Thus, biochemical mechanisms in the vesicle membrane can be studied in their natural intracellular environment rather than in vesicles isolated in vitro in which retention is impaired. Differences in responses have already been reported. In vitro, ATP stimulates either uptake or release of NE in isolated vesicles depending upon the presence of PO_4^{3-} or Cl^-, respectively. In the natural environment, ATP apparently stimulates uptake although [Cl^-] is essentially physiological. Moreover, the stimulation of uptake of NE by ATP is energized by H^+ transport, but that apparently does not account for total uptake.

Proposed Course of Project: It is hoped that some of the projects planned for this past year will be started and completed in the coming year. Studies of the effects of metabolic inhibitors, transport blockers and other agents which inhibit the establishment or dissipation of proton gradients will continue. We will continue our efforts to obtain more electronmicrographic examples, as well as pharmacological evidence of fused or attached vesicle and plasma membranes and to observe other significant features of the morphology of normal and stimulated nerve endings. We have found an example of fused membranes, possibly with elimination of layers. By inducing a sustained period of rapid secretion, it is thought that Ch^+-Ca^{++} stimulation will increase the probability of our finding more examples of fused vesicle and plasma membranes. Attempts to perform our own experiments on the pharmacologically or electrically driven release of neuronal ^{3}H-NE will be made.

Publications:

Bogdanski, D.F.: Monovalent cation dependency for the inhibition of outward transport of [^{3}H]norepinephrine. Neuropharmacology 24: 13-18, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01850-16 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biochemistry of the Spontaneously Hypertensive Rat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Martina Diolulu Research Fellow HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

Membrane fractions enriched in sarcoplasmic reticulum (SR) were isolated from the cardiac ventricles of 10-month-old, stroke-prone spontaneously hypertensive rats (SHRSP) which had been maintained for nine months on one of four experimental diets: low protein (LP) (19% protein), standard (STD) (24% protein), high protein (HP) (32% protein), or high methionine (1.9% methionine) (MET). ATPase activities, as well as ATP-dependent Ca2+ binding and Ca2+-uptake activities, of the isolated SR were determined to examine the influence of diet on myocardial Ca2+-pump activity. SR from all four groups exhibited similar Mg2+-ATPase activity. However, the (Ca2+ + Mg2+)-ATPase activity was significantly elevated in SR from rats on the MET diet while the activity in the other groups showed no significant differences. After 15 sec of incubation, Ca2+-uptake (presence of oxalate) in SR from the LP group was significantly less than Ca2+-uptake in SR from each of the three other diet groups. Ca2+ binding (absence of oxalate) in the SR from the LP group was also significantly less than that from each of the three other diet groups. Kinetic analysis of SR Ca2+-uptake over 60 sec revealed that the Bmax of the MET group was significantly higher than Bmax of the STD diet group. In addition, the Bmax of the LP group was significantly lower than Bmax of the HP and MET groups. There was no significant difference in affinity of the SR Ca2+-uptake system among the four diet groups. These results indicate that modification of dietary protein can influence myocardial SR Ca2+-pump function.

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In recent years, several investigators (Ito, J. Mol. Cell Cardiol. 4: 507-517, 1972; Suko, BBA 252: 324-327, 1971) have attempted to relate pathological changes in heart function to alterations in the cardiac sarcoplasmic reticulum. Defective calcium handling by vascular smooth muscle membranes has also been shown to occur during the prehypertensive stage in spontaneously hypertensive rats (SHR) (Kwan, In Vasodilation, Vanhoutte, P.M., ed., Raven Press, pp. 405-415, 1981a). The increase in resistance in hypertension could result from supersensitivity to vasoconstrictor stimuli or from altered vascular structure. Calcium is believed to be part of the trigger mechanism of vasoconstriction; hence, a derangement in Ca^{2+} metabolism could be a cause of vascular hyperreactivity (Weis, Acta Physiol. Scand. Suppl. 409: 5-58, 1974). The trigger for contraction of all muscle types (skeletal, cardiac and smooth) is an increase in cytosolic calcium ion above 10^{-7} M concentration. In the muscle, two different ATP-dependent Ca^{2+}-transport systems are operative, the sarcolemmal pump and the sarcoplasmic reticulum Ca^{2+} pump. These complex membrane systems serve as a sink for Ca^{2+} ions during muscle relaxation and as a Ca^{2+} source during muscle excitation (Hasselbach, Prog. Biophs. Mol. Biol. 14: 167-222, 1965; Tada, Physiol. Rev. 58: 1-79, 1978).

Subcellular fractions enriched in plasma membrane and sarcoplasmic reticulum isolated from spontaneously hypertensive rats (SHR) have been shown to exhibit reduced ATP-dependent Ca^{2+} accumulation (Aoki et al., Jap. Circ. J. 38: 1115-1120, 1974). Studies from our laboratory and others have demonstrated that in SHR a high protein diet or a high fat diet containing a moderate amount of protein significantly retards the development of stroke and reduces the accompanying incidence of cerebral and cardiovascular lesions, while a low protein diet accelerates the disorders (Yamori et al., Hypertension 6: 49-53; Lovenberg and Yamori, Clin. Exp. Hyperten. Theory and Practice A6 (1&2): 417-426, 1984). Such diets also tend to marginally lower blood pressure in SHR. The objective in the present study was to examine the effect of different diets on several biochemical parameters of ventricular sarcoplasmic reticulum isolated from stroke-prone spontaneously hypertensive rats. The results are interpreted in relation to the possible protective mechanisms of a high protein diet.

Methods:

Animals:

Forty male stroke-prone spontaneously hypertensive rats (SHRSP/A3N) 4 weeks of age were obtained from the Small Animal Section at the National Institutes of Health. The rats were divided into four equal groups of 10 rats such that each group was randomly assigned to one of the four experimental diets. Low protein (LP) (19% protein), standard (STD) (24% protein), high protein (HP) (32% protein), or high methionine (1.9% methionine) (MET). During the 9-month duration of this experiment, rats were housed in conventional facilities maintained on a 12-hour day-night cycle with a mean room temperature of 25°C. The rats were caged in plastic cages, five rats per cage. Rats had ad libitum access to the appropriate diet and clean drinking water. At the end of nine months (when the rats were 10 months of age) the blood pressure of each rat was measured by a tail cuff plethysmographic technique, body weights were recorded, and each rat was sacrificed by decapitation. Hearts were quickly excised for the biochemical assays.

Preparation of Sarcoplasmic Reticulum (SR): The excised hearts were trimmed of atria, and the ventricles thoroughly rinsed in ice-cold oxygen-bubbled Krebs-Ringer solution (pH 7.4) in which EGTA was substituted for Ca^{2+}. The ventricles were then blotted dry, weighed, and chopped into small pieces with scissors.

Because cardiac preparations are extremely labile resulting in a poor yield of SR, cardiac microsomes enriched in SR were prepared by a modification of the method of Harigaya and Schwartz. Preparative procedures were carried out at $4^{o}C$. Chopped ventricles were homogenized for 20 sec with a Polytron at a setting of 5 in 4-5 volumes of ice-cold medium consisting of 0.1 M KCl, 25 mM Tris-maleate buffer (pH 6.8) and 1 gm/200 ml of α-tocopherol. α-Tocopherol was included as a lipid antioxidant to protect microsomes from hematin-catalyzed peroxidation of lipid during homogenization. However, in preliminary experiments no differences in Ca^{2+} uptake activities were observed in SR fractions prepared in the presence and absence of α-tocopherol. The homogenate was passed through four layers of cheese cloth to remove coarse material and centrifuged in a refrigerated Sorvall RC-2 centrifuge at 600 X g for 20 min to remove myofibrils, nuclei, and debris. The resulting supernatant fraction was saved. The sediment was resuspended in 25 mM Tris-maleate buffer, homogenized again for 10 sec at a setting of 5, and sedimented again at 600 X g for 20 min. This process was repeated 3 times and the combined supernatant fractions were centrifuged at 8700 X g for 30 min to remove mitochondria. The pellet was discarded and the supernatant centrifuged at 37,000 X g for 50 min to sediment the crude SR. The resulting pellet was resuspended in 10 ml of 0.6 M KCl and incubated for 30 min at $4^{o}C$ to solubilize any contaminating actomyosin. The suspension was again sedimented at 37,000 X g for 50 min. Finally, the SR pellet was suspended in Tris-maleate buffer containing 40% sucrose and stored at $-70^{o}C$ until assayed. Rigorous electron microscopic examination of all SR fractions showed that the preparations consisted of membrane vesicles relatively uniform in size and almost completely devoid of intact mitochondria or of recognizable fragments of mitochondria. In addition, no significant differences were observed in Ca^{2+} uptake experiments carried out in the absence and presence of NaN_3 (metabolic inhibitor) (data not shown) indicating negligible mitochrondrial contamination.

Biochemical Determinations: Cardiac SR Mg^{2+}-ATPase and (Mg^{2+} + Ca^{2+})-ATPase activities were determined by monitoring liberated inorganic phosphate. The reaction mixture contained 120 mM KCl, 5 mM $MgCl_2$, 2.5 mM ATP, 25 mM Tris-maleate buffer (pH 6.8), 0.09-0.13 mg SR protein, and 0.1 mM $CaCl_2$ for (Mg^{2+} + Ca^{2+})-ATPase activity or 0.1 mM EGTA for Mg^{2+}-ATPase activity in a total volume of 1.5 ml. The reaction mixtures were incubated at room temperature ($25^{o}C$). After the appropriate time, the reactions were stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA). Inorganic phosphate liberated was measured by the method of Fiske and SubbaRow. Protein was assayed according to the method of Bradford.

Ca^{2+} Binding and Uptake: Measurement of Ca^{2+} binding (steady state) and uptake activities of cardiac SR was carried out with $^{45}Ca^{2+}$ and Millipore filtration technique as described by Narayanan with minor modification. For Ca^{2+} binding assays, the reaction mixture contained 25 mM Tris-maleate (pH 6.8), 5 mM $MgCl_2$, 2.5 mM ATP, 120 mM KCl, 5 mM NaN_3, and varying concentrations of $CaCl_2$ labelled with 0.1 μCi/ml $^{45}CaCl_2$ in a total volume of 1.0 ml. The free calcium ion

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concentrations in the assay medium were calculated as described by Katz et al. The assay was initiated at room temperature (25°C) by the addition of the SR fraction (0.09-0.125 mg/ml) into the incubation medium. The NaN_3 was included in the incubation medium to abolish accumulation of Ca^{2+} by any mitochondria that might be present in the preparation. At the desired times of incubation (15 sec-90 sec), the reaction was terminated by rapidly filtering the sample mixture with vacuum through prewashed Millipore filters (0.45 μM pore size, 25 mm diameter) that were presoaked in 100 μM $CaCl_2$ solution to reduce non-specific binding of $^{45}Ca^{2+}$. After filtration, the filters were washed again three times with 2 ml of assay medium containing 0.1 mM EGTA but no $^{45}Ca^{2+}$. The filters were transferred to scintillation vials, and the radioactivity determined by liquid scintillation spectrometry. Appropriate controls without SR membranes and/or ATP were included in all experiments.

For the determination of Ca^{2+} uptake, assays were carried out as described above except that the incubation medium contained 2.5 mM potassium oxalate in addition to the other components. Thus, the terms "Ca^{2+} binding" and "Ca^{2+} accumulation" used in this report denote Ca^{2+} uptake measured in the absence and presence, respectively, of oxalate in the incubation medium. The term Ca^{2+} binding or steady-state Ca^{2+} uptake in this report embraces all calcium bound to the SR vesicles extracellularly and intracellularly, as well as calcium free within the intraventricular space. As in many other microsomal Ca^{2+}-uptake systems, Ca^{2+} uptake in SR is enhanced by the presence of oxalate which serves as a trapping agent for the accumulated cation. Kinetic constants were calculated by Scatchard analysis.

Statistical Analysis of Data: Experimental data were compared by analysis of variance followed by Student's t-test when appropriate. All results are expressed as mean ± s.e.m. with N = 5 determinations (each with SR pooled from two animals) per group.

Major Findings: ATPase activities, as well as ATP-dependent Ca^{2+} binding and Ca^{2+}-uptake activities, of the isolated SR were determined to examine the influence of diet on myocardial Ca^{2+}-pump activity. SR from all four groups exhibited similar Mg^{2+}-ATPase activity. However, the (Ca^{2+} + Mg^{2+})-ATPase activity was significantly elevated in SR from rats on the MET diet while the activity in the other groups showed no significant differences. After 15 sec of incubation, Ca^{2+}-uptake (presence of oxalate) in SR from the LP group was significantly less than Ca^{2+}-uptake in SR from each of the three other diet groups. Ca^{2+} binding (absence of oxalate) in the SR from the LP group was also significantly less than that from each of the three other diet groups. Kinetic analysis of SR Ca^{2+}-uptake over 60 sec revealed that the B_{max} of the MET group was significantly higher than B_{max} of the STD diet group. In addition, the B_{max} of the LP group was significantly lower than B_{max} of the HP and MET groups. There was no significant difference in affinity of the SR Ca^{2+}-uptake system among the four diet groups. These results indicate that modification of dietary protein can influence myocardial SR Ca^{2+}-pump function.

Significance to Biomedical Research: Hypertension is a major health problem in the world. Total pharmacological control of hypertension presents many problems in terms of cost and unknown long-term effects of pharmacological agents. The research in this project is designed to better understand the role of diet in

blood pressure regulation. While it is known that dietary protein as specific amino acids may lower blood pressure, the findings of the project provide one of the few clues as to the biochemical basis for this phenomenon.

Proposed Course of Project: The aim of the present study is to investigate further the influence of diet on possible defects in the coupling of calcium control through protein phosphorylation in vascular smooth muscle in genetically-hypertensive animals. Smooth muscle proteins will be separated and characterized by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and analyzed by autoradiography. Specifically, we are interested in a) determining whether or not phospholamban is phosphorylated and whether there are differences in the SHR vs normotensive rats, b) determine the influence of dietary manipulation on phospholamban phosphorylation. Phospholamban is an SR protein whose phosphorylation regulates Ca^{++} transport mediated by a Ca^{2+}-dependent ATPase activity, and c) since the concentration of ionized intracellular calcium is the rate-limiting determinant of phosphorylation reactions, and hence the physiologic regulator of muscle contraction, this study also intends to determine the content of Ca^{2+} in vascular smooth muscle of SHR vs normotensive and the influence of diet.

Publications:

Lovenberg, W.: Possible relationship between nutrition and cardiovascular disease in experimental animals. In Yamori, Y. and Lovenberg, W. (Eds.): Nutritional Prevention of Cardiovascular Diseases. Academic Press, New York, 1984, pp. 21-26.

Lovenberg, W. and Yamori, Y.: Dietary Protein, the central nervous system and hypertension. Annals of Internal Medicine, in press.

Diolulu, M.A., Buck, S.H., Knapka, J., and Lovenberg, W.: ATP-dependent calcium uptake in myocardial sarcoplasmic reticulum from spontaneously hypertensive rats: Effect of modification of dietary protein. Clin. Exp. Hypertension, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01879-08 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanism for Receptor Regulation during Sub- and Supersensitivity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
Others:	Y. Kataoka	Staff Fellow	PP NIMH
	E. Sanna	Guest Worker	HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	0.5	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Canine adrenal glands served as a model to study neurotransmitter release elicited by GABA receptor stimulation. Infusion of GABA or GABA-mimetics in the adrenal gland increased the release of catecholamines and met-enkephalin-like peptides. This release was a consequence of direct depolarization of chromaffin cell membranes and was not mediated transsynaptically through acetylcholine or met-enkephalin.

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GPO 914-918

Objectives: With the advent of techniques to label neurotransmitter recognition sites, much progress has been made in studying the dynamic state of neurotransmitter receptors. Prolonged occupancy or deprivation of recognition sites by or from specific ligands was shown to cause receptor sub- or supersensitivity, respectively. Recent discoveries that more than one neuromodulator coexist in the same neuron and can be coreleased introduces the new concept that more than one chemical signal may be involved in transmitting interneuronal communication. In corpus striatum multiple receptive sites for dopamine were shown to exist on cholinergic neurons and possibly also on GABA-ergic neurons. Increased dopaminergic transmission results in depolarization of the striatal cholinergic neurons, while activation of GABA-ergic neurons hyperpolarize the post-synaptic cell membrane. Furthermore, in striatum met-enkephalin-receptors and post-synaptic D2 receptors have similar effect on acetylcholine release and their recognition sites appear to be coupled to adenylate cyclase through the inhibiting subunit of G/F protein. The goal of the present project is to establish a simple and suitable model to study the interrelationship between various neurotransmitter in regulating subsequent physiological events. The role of acetylcholine and GABA receptors in the regulation of neurotransmittter release was studied in adrenal chromaffine cells.

Methods:

Studies of neurotransmitter release include measurements of acetylcholine release from striatal slices and adrenaline release from primary cultures of chromaffin cells, adrenal/noradrenaline, and met-enkephalin release elicited by splanchnic nerve stimulation or various drugs is measured in vivo in effluent blood obtained by cannulating the lumbar adrenal vein in dogs.

Measurement of cyclic AMP formation are carried out by radioimmunoassay kit. $^{45}Ca^{2+}$-uptake in striatal slices is measured in presence and absence of veratridine.

Major Findings: GABA is present in adrenal medullary nerve fibers and chromaffin cells. Moreover, GABA-receptors are present in adrenal chromaffin cell membranes. GABA and GABA-mimetic drugs release catecholamines and met-enkephalin-like peptides into the circulation. This release is not blocked by hexamethionium, naloxone or splanchnicotomy, but can be attenuated by pretreatment with bicuculline. These data suggest that the GABA-elicited neurotransmitter release is a consequence of chromaffin cell depolarization caused by direct stimulation of GABA receptors and does not involve transsynaptic mechanism mediated by acetylcholine or met-enkephalin.

Significance to Biomedical Research and Institute Programs: This research project focuses on cholinergic and GABA-ergic neurotransmitter receptors on adrenal chromaffin cell membranes that regulate the release of catecholamines and met-enkephalin-like peptides from adrenal glands into the blood circulation. Our findings demonstrate the existense of GABA receptors on chromaffin cell and show that GABA receptors are operative in releasing neurotransmitters into the circulation by direct depolarization of chromaffin cell membranes. These findings may lead to the development of a new type of drug that modulate adrenal medullary function without modifying splanchnic nerve activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03511-06 HE

PERIOD COVERED: October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*: The Role of the Brain Serotonergic in Blood Pressure Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principel Investigator.) (Name, title, leboratory, and institute affiliation)*

PI:	William A. Wolf	Guest Worker	HE NHLBI
Others:	Donald M. Kuhn	Pharmacologist	HE NHLBI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Hypertension-Endocrine Branch

SECTION: Biochemical Pharmacology

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL: 0.3	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The role of the serotonergic neuronal system on blood pressure control of normotensive WKY rats and spontaneously hypertensive rats (SHR) was studied by injecting L-tryptophan, the metabolic precursor to brain serotonin. It was observed that the dose- and temporal effects of tryptophan on blood pressure were not related to the effects of tryptophan on serotonin levels throughtout the brain. The dextro-rotary isomer of tryptophan was no different from L-tryptophan in its ability to increase brain serotonin but D-tryptophan does not alter blood pressure in SHR or WKY. An analog of tryptophan, TR 3369, wherein the carboxyl group has been moved from the alpha to the beta carbon of the side chain, dramatically lowers blood pressure in the SHR without influencing 5-HT neurochemistry. Other peripheral metabolites of tryptophan including kynurenin and kynuramine, also lower blood pressure. Taken together, these results indicate that the cardiovascular effects of L-tryptophan are not mediated by brain serotonin and may result from direct effects of the amino acid itself or a primary metabolite on the vasculature.

557

Objectives: The role of the brain serotonin system in blood pressure regulation is not well understood (Kuhn et al., Hypertension, 1980, 2, 243-255). This is due in part to the difficulty in specifically manipulating the central serotonin system by pharmacological means. To circumvent this problem, investigators often inject L-tryptophan, the precursor amino acid for serotonin synthesis. Presumably, tryptophan is converted to serotonin only within those cells containing the required enzymes (tryptophan hydroxylase and aromatic L-amino acid decarboxylase) and the newly synthesized 5-HT is released into the synapse where it exerts its physiological effects. The objective of this study was to assess the effects of L-tryptophan on blood pressure of normotensive and hypertensive rats and to correlate changes in serotonin neurochemistry to changes in blood pressure.

Methods: Male Wistar-Kyoto and spontaneously hypertensive rats were housed in temperature and humidity controlled isolator chambers. Blood pressure was measured by a tail cuff method which uses a photoelectric sensor to detect tail pulses. The method is used at ambient temperature unlike all other indirect tail cuff methods where it is required to heat animals in order to dilate tail vessels. Additional studies with TR 3369, kynuranine, and kynuramine were done in animals with indwelling catheters.

Major Findings: Injections of L-tryptophan increased blood pressure in the WKY. Doses of 25, 50, and 100 mg/kg each increased blood pressure by 10-15 mm Hg. This effect was observed within 30 min of tryptophan injections and blood pressure returned to normal by 4 hours. On the other hand, SHRs showed little response to 25 and 50 mg/kg of L-tryptophan but responded with substantial reductions (7-30 mm Hg) in blood pressure at 30, 60, and 120 min after injection of 100 mg/kg. Neurochemical analysis indicated that brain serotonin levels were increased uniformly by approximately 1.2-1.4 fold while brain tryptophan levels increased over 10 fold at the highest dose. The dose- and temporal effects of L-tryptophan on blood pressure were not related to the effects of L-tryptophan on serotonin neurochemistry. For example, 100 mg/kg of L-tryptophan increased brain serotonin no more than doses of 25 or 50 mg/kg but this dose increased blood pressure in WKY and decreased blood pressure in SHR. Furthermore, injections of equimolar doses of D-tryptophan increased brain serotonin and 5-hydroxindoleacetic acid to the same extent as L-tryptophan in the SHR, but D-tryptophan does not alter blood pressure.

TR 3369 was injected into SHR's since this compound is a close structural analog of tryptophan. However, it cannot enter the 5-HT biosynthetic pathway since it is not a substrate for either tryptophan hydroxylase or aromatic L-amino acid decarboxylase. Nevertheless, TR 3369 produced a significant antihypertensive effect in SHRs at a dose of 10 mg/kg. Similarly, kynuramine and kynuranine lowered blood pressure rapidly but transiently in SHR. It appears that these agents caused vasodilation since the pulse pressure was not of sufficient intensity to be detected plethysmographically. Direct arterial blood pressure recording confirmed that TR 3369, kynuramine, and kynuranine lowered blood pressure in SHRs.

Significance to Biomedical Research and Institute Program: The pathophysiology of hypertension is poorly understood yet some alteration in the functioning of

the nervous system could contribute to the genesis or maintenance of hypertension. In order to study the neurochemical influence upon blood pressure and todevelop pharmacological modalities which correct this dysfunction, one must fully understand the mechanism of action of antihypertensive agents which are thought to exert their primary effects via the brain. L-tryptophan has mild antihypertensive effects and it is generally assumed that the physiological effects of tryptophan are mediated by increased serotonin synthesis and release consequent to tryptophan administration. The present experiments reveal that the antihypertensive effects of L-tryptophan are not mediated by brain serotonin and, if anything, indicate a peripheral site of action for the amino acid. The high doses of tryptphan needed to lower blood pressure suggest further that its influence on blood pressure is secondary. If serotonin could be of some use in lowering blood pressure, and it could, injections of its precursor tryptophan or dietary supplementation with tryptophan do not appear to represent a reasonably potent and specific enough means by which serotonin can be altered in a functional sense. Finally, apart from an application in hypertension research, the present results are leading to a re-evaluation of a number of physiological processes once thought to be dependent on brain serotonin.

Proposed Course of Project:

1. Study whether peripheral serotonin antagonists will influence the cardiovascular effects of L-tryptophan and TR 3369.

2. Investigate the mechanism by which L-tryptophan and metabolites alter blood pressure by testing these agents in various organ bioassays.

3. Investigate the effects of 5-hydroxytryptophan on blood pressure in SHR and WKY. This amino acid is another precursor of serotonin which is frequently used to alter brain serotonin.

Publications:

Kuhn, D.M., and Lovenberg, W.: Tryptophan hydroxylase. In Blakely, R.L., and Benkovic, S. (Eds.): Chemistry and Biochemistry of Pterins. Wiley, New York, 1985, pp. 353-382.

Wolf, W.A., and Kuhn, D.M.: Effects of L-tryptophan on blood pressure in normotensive and hypertensive rats. J. Pharmacol. Exp. Ther. 230: 324-329, 1984.

Wolf, W.A., Lovenberg, W., and Kuhn, D.M.: Serotonin and central regulation of blood pressure. In P.M. Vanhoutte (Ed.): Serotonin and the Cardiovascular System. New York, Raven Press, 1985, pp. 63-75.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03513-06 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular components of the striatal dopamine uptake system

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
Others:	Cristina Missale		Univ. Brescia
	Ulrike Berresheim	Guest Scientist	HE NHLBI

COOPERATING UNITS *(if any)*
Institute of Pharmacology, University of Brescia, Brescia, Italy

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	0.5	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In corpus striatum, Na+-dependent 3H-cocaine binding can be used as a biochemical marker for dopamine uptake sites located in presynaptic dopaminergic terminals. A GABA-modulin-like polypeptide purified from striatal synaptosomes reduces Na+-dependent 3H-cocaine binding to striatal membrane preparations.

560

GPO 914-918

Objectives: Previous studies on the specific binding of ^{3}H-cocaine, ^{3}H-mazindol, and ^{3}H-methylphenidate suggest that these radioligands bind to a site in striatal membranes that is associated with the dopamine uptake system since these ligands were shown to be potent blockers of ^{3}H-dopamine uptake. Therefore, the use of ^{3}H-cocaine as a probe for labelling dopaminergic nerve endings may be relevant to study mechanisms that regulate uptake, release, and synthesis of dopamine. Further, recent data showing that daily injections of cocaine for 3 weeks cause an increase in ^{3}H-cocaine recognition sites suggest the existence of an endogenous ligand.

Methods: High affinity binding of ^{3}cocaine was measured as described by Kennedy and Hanbauer (J. Neurochem. 41, 172-178, 1983).

^{3}H-Dopamine uptake into brain slices was measured in presence 100 µM pargyline, ascorbic acid in Krebs solution equilibrated with 95% O_2/5% CO_2. Non-specific ^{3}H-dopamine uptake was determined in presence of 100 µM nomifensine.

Isolation of endogenous ligand from brain included extraction of brain synaptosomes with 1 M CH_3 COOH at 90°C, filtration of the supernatant fraction on Biogel P-10, and further purification on HPLC (p-Bondapak ^{18}C-column, Waters Inc.).

Major Findings: Studies of the effect of cocaine or nomifensine on dopamine uptake showed that dopamine uptake is differentially regulated in corpus striatum and nucleus accumbens. ^{3}H-Dopamine uptake in striatal slices is decreased by 50% in presence of 10 µM cocaine, while in slices of nucleus accumbens half-maximal inhibition is caused by 100 µM cocaine. Injection of cocaine (20 mg/kg, i.p.) reduces ^{3}H-dopamine uptake in striatal slices, but increases ^{3}H-dopamine uptake in slices of nucleus accumbens. Furthermore, a Na^+-dependent population of ^{3}H-cocaine binding sites is present in crude synaptosomal membranes of corpus striatum, but is not present in membranes prepared from nucleus accumbens.

Na^+-dependent ^{3}H-cocaine binding is displaced by a polypeptide with a molecular weight of 17.5 K and pI=11.4. Its amino acid composition is identical to that of GABA-modulin. In fact, a similar extent of inhibition of ^{3}H-cocaine binding was obtained by purified GABA-modulin and the polypeptide isolated from striatal synaptosomes.

Proposed Course of Project: Future research is aimed to study the biochemical mechanisms underlying the different regulation of ^{3}H-dopamine uptake in corpus striatum and nucleus accumbens. Future studies are planned to determine whether GABA-modulin is present in dopamine-rich brain areas such as corpus striatum and nucleus accumbens and whether its function can be linked to a receptor at the presynaptic dopaminergic terminals that shows high affinity for dopamine uptake blockers and that may be operative in the regulation of dopamine uptake.

Significance to Biomedical Research and Institute Programs: This ongoing research project will improve the understanding of the mechanism underlying dopamine uptake. Knowledge on the mechanism of interaction of dopamine uptake blockers with specific membrane sites responsible for dopamine uptake possesses

therapeutic potential in disease states associated with altered dopaminergic transmission.

Publications:

Hanbauer, I., Kennedy, L.T., Missale, M.C., and Bruckwick, E.C.: Cocaine binding sites located in striatal membranes are regulatory sites for dopaminergic synapsis. In Biggio, G., Spano, P.-F., Toffano, G., and Gessa, G.L. (Eds.): Adv. in Bioscience: Neuromodulation and Brain Function. Pergamon Press, 1984, pp. 41-49.

Hanbauer, I., and U. Berresheim: Endogenous modulation of striatal dopamine uptake. Clinical Neuropharmacology 7 (Suppl. 1): 796-797, 1984.

Missale, C., Castelletti, L., Govoni, S., Spano, P.F., Trabucchi, M., and Hanbauer, I.: Dopamine uptake is differentially regulated in rat striatum and nucleus accumbens. J. Neurochem. 45: 51-56, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03516-06 HE

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biosynthesis, Distribution and Biological Role of Substance P and Its Receptors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Mei-Lie Swenberg	Research Chemist	HE NHLBI
Other:	Stephen Buck	PRAT Fellow	HE NHLBI
	Rita Liu	Ass. Professor	USUHS
	Candace Pert	Section Chief	NSB NIMH

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Localization of SP receptor and SP immunocytochemistry were conducted for the whole rat embryo of 14, 17, and 20 days of gestation and the new born on the day of birth. Both CNS and peripheral system was well developed as early as 17 day of gestation.

Based on the membrane binding studies with 3HSP, the number of binding sites increased during the development till the first 4 weeks after birth when the brain size reached maturity, it decreased afterward.

Purified SP membrane receptor from rat embryo brain was subjected for dissociation of subunit without enzymatic cleavage and iodination with Bolten Hunter reagent.

Two different brain areas of mature young rat (brain stem and midbrain plus olfactory bulb) were subjected to Percol gradient separation. Two fractions (#6 & 8) of synaptosome isolated from the two area exhibited distinctive difference in 3HSP binding this might indicate the possible functional and chemical difference of the two fractions.

Antibody against SP antibody has been initiated on New Zealand white rabbits.

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Objectives: Substance P (SP) appears to serve a role as a neurotransmitter and neuromodulator in both the CNS and periphery. It seemed important to know more about how this neuropeptide interacts with its receptor. In order to achieve this, there are several approaches that could be pursued, these include: 1) extraction, purification and characterization of the receptor molecule, 2) derivation of new probes for studying the interaction of the peptide with its receptor, such as an antiidiotypic antibody, 3) determination of the developmental pattern for SP and its receptor and correlation of this with changing function, and 4) since it is a major neurotransmitter in the primary sensory system, study on the primary sensory system might be another approach. These types of experimentation should lead to a better understanding of the role of SP.

Methods: The receptor characteristics of rat brain at various stages of development were conducted with ^{3}H-SP in the presence of Kreb salts and 20 mM Tris buffer.

Localization of SP receptor in whole embryonic slices were performed with Bolten Hunter iodinated SP (BHSP) as described by Helke et al. (Neurosci. 12: 215-223, 1984). SP immunocytochemistry was performed by Biotin-peroxidase method.

The SP receptor solubilized in a buffer containing triton X-100 or CHAPSO was purified by a wheat germ lectin-Sepharose column chromatography and elution with 0.2 N N-acetylglucosamine. Dissociation of subunits were conducted in SDS and mercaptoethanol buffer for 10 min in boiling water bath.

Molecular weight and homogeneity of protein preparations were determined with a PAA electrophoretic system. Thin layer isolectric focussing was used to measure the isolectric point of proteins.

Major Findings:

1) SP Receptor Isolation. Two subunits of molecular weight less than 400 K dalton were obtained from the isolated brain membrane receptor after the treatment with mercaptorethanol SDS and boiling. It appeared that receptor of embryonic brain is more susceptible to Bolten Hunter iodination than that of postnatal brain. There might be chemical differences during the development.

2) SP receptor localization in prenatal rat BHSP binding was dense as observed in spinal cord, brain stem, lining of stomach, intestine, and surface of toungue after 14 day of gestation. There were difference betwen the binding studies on membrane pellet with ^{3}H-SP and those of BHSP in localization.

3) Immunocytochemical result indicated well established SP nerve fiber and cells as early as 14 day gestation, both central and peripheral system. SP distribution is observed in muscular system near the birth and increase after birth.

4) Development of SP receptor in brain increased during the prenatal development and during the first few weeks (3-4 wk) of life. After the brain reachs maximum growth, the B_{max} reduced after 4 weeks while the SP content stayed up to maturation (200 g body wt 6 week). Ions Ca^{++}, Mg^{++}, K^{+}, and Na^{+} were found essential

to obtain sturation in ^{3}H-SP binding on rat brain membrane. Extensive washing of the membrane seemed to increase binding and inhibit the obtaining saturation.

5) Binding studies on the two frozen synaptosomal pellet obtained by Percol gradient from rat (190 ± 20 g) brain indicated striking difference in binding activity per gm protein. Binding of fraction 8 (15% Percol fraction) of higher density was more than two fold greater than that of fraction 6 (10% Percol fraction) and it was found the similar result in different brain area. This might show the specific characteristic and functional difference of the two fraction of synaptosome.

6) SP and SP receptors were localized on tounge papilli.

7) Subunits of anti-SP antibody raised, isolated, and purified in our laboratory were determined as 24.8 and 48.5 K dalton and native immunoglobulin of estimated 293 K dalton.

Significance to Biomedical Research and Institute Programs: The peptide substance P appears to be an important neurotransmitter in the CNS. Previous studies in our laboratory have demonstrated the nature of the interaction of this neuronal system with the dopamine and serotonin neuronal systems. The current experiments were designed to further understand both the development of the SP system and its interaction with the endocrine system in CNS and peripheral system. The work is directed at understanding the role of SP in cardiovascular control and other physiologic systems.

Proposed Course of Project:

1) With isolated receptor available, antibody could be prepared for use in the study of regulation and function of SP in vascular and sensory nervous system as well as in neuroendocrine system.

2) Hybridoma cell line will be obtained to produce antibodies against isolated SP receptor subunit of CNS and peripheral.

3) Continue the study of a possible new function of SP in prenatal development and possibility of deriving methodology in preventive medicine. Studies on earlier stage (before 14 day of gestation) in development is required to establish the oncology of initiation of SP neuron and receptor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03520-05 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Dopamine Receptor Regulation in Schizophrenic Illness

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
Other:	Michael Jennewein	Guest Scientist	HE NHLBI
	Enrico Sanna	Guest Scientist	HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The functional efficiency of the G/F protein that facilitates adenylate cyclase activation is increased in dopamine-rich brain areas of schizophrenic subjects. These findings suggest that aberrant dopaminergic transmission in brain of schizophrenics could be due to a basic defect in the function of the G/F protein. Studies on the effect of BHT-920, a compound which has been shown to be therapeutically effective in schizophrenic subjects, showed that it decreased 3H-spiroperidol binding and it reduced tyrosine hydroxylase activity in caudate nucleus.

Objectives: The most consistent neurochemical finding relating to schizophrenia has been an increased density of D-2 dopamine receptors and an increased responsiveness to NaF and GppNHp of adenylate cyclase in post-mortem dopamine-rich brain tissue from schizophrenics. While the increased responsiveness of adenylate cyclase indicates changes occuring at the post-synaptic dopamine receptor, the increased number of D-2 receptors suggest that alterations may occur both at pre- and post-synaptic sites. To obtain further information in support of this working model, studies of pre- and post-synaptic processes linked to dopaminergic neurotransmission are required. Recent reports on the compound BHT 920 showed that it was the effect of BHT 920 on therapeutically effective when given to schizophrenic subjects. Therefore, various presynaptic dopaminergic processes were studied to obtain further clues for the dopamine hypothesis in schizophrenia.

Methods:

Photoaffinity labeling of G/F protein: Crude synaptosomal pellets were extracted with 50 mM Tris buffer pH 7.4 containing 1% n-octylglucoside. To the solubilized protein extract ^{32}P-8-azido GTP was added and the solutions were exposed to UV for 2 min at room temperature. As control for non-specific labeling served not UV-exposed assay mixtures.

Studies on pre-synaptic dopaminergic processes: The effect of BHT 920 on ^{3}H-dopamine uptake, Na^{+}-dependent ^{3}H-cocaine binding, ^{3}H-spiroperidol binding, and tyrosine hydroxylase activity were studied in various brain areas and adrenal glands of rats.

Major Findings:

1) Photoaffinity labeling of crude synaptosomes prepared from caudate nucleus of schizophrenic, or non-schizophrenic, showed an increase binding of ^{32}P-GTP to protein bands with molecular weights of 52,000 and 43,000 in schizophrenic subjects.

2) Studies on BHT 920 carried out caudate nucleus of rats showed that this compound displaces ^{3}H-spiroperidol from its specific binding sites and that it significantly reduces tyrosine hydroxylase activity. BHT 920 failed to alter ^{3}H-dopamine uptake in striatal slices or the Na^{+}-dependent ^{3}H-cocaine binding to crude striatal synaptosomal membranes. The decrease of tyrosine hydroxylase activity elicited by subcutaneous injection of BHT 920, occured specifically in caudate nucleus because it failed to change enzyme activity in superior cervical ganglion or adrenal medulla of the same rat.

Proposed Course of Action: Reports in the literature indicated that neuroleptics with therapeutic effect on the negative symptoms of schizophrenia decreased ^{3}H-nitrendipine binding, a marker for Ca^{2+} channels. Studies on radioligand binding of Ca^{2+} channel antagonists are in progress that will allow us to determine whether the function of voltage-dependent Ca^{2+} channels is altered in schizophrenic brain.

Significance of Biomedical Research: This research project is designed to obtain a better understanding of biochemical defects that may exist in

schizophrenic illness. Better insight on basic biochemical mechanisms linked to dopaminergic transmission will be studied on the level of pre- and postsynaptic processes and will provide a basis for a more specific and effective therapy.

Publications:

Hanbauer, I., Memo, M., and Kleinman, J.E.: Increased efficiency in the coupling of dopamine-D1 recognition sites with adenylate cyclase in dopamine-rich brain areas of schizophrenics. In: Schizophrenia: an Integrative View. John Libbey Publisher, London (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03536-03 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Carboxylmethylation of Calmodulin-Binding Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Walter Lovenberg	Laboratory Chief	HE NHLBI
Others:	Melvin L. Billingsley	Staff Fellow	HE NHLBI
	Randall Kincaid	Staff Fellow	CM NHLBI
	Paul Velletri	Staff Fellow	HE NHLBI
	Donald Kuhn	Pharmacologist	HE NHLBI

COOPERATING UNITS *(if any)*
Laboratory of Cellular Metabolism, NHLBI

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Protein-O-carboxylmethyltransferase (PCM) catalzes the transfer of a methyl group from S-adenosylmethionine to aspartyl residues on a variety of proteins. The function of this enzyme is not known, although it has been postulated to participate in the repair of defective proteins. It has also been suggested that it may participate in regulatory events for other enzymes. Previous work in our laboratory had shown that calmodulin binding proteins of a particularly good substrates for PCM and the activity of enzymes such as the calcium-dependent phosphodiesterase, calcineurin, and calmodulin-dependent protein kinase do indeed appear to be regulated by the methylation reaction. Using the expertise developed in this project, a new approach to the detection of binding proteins has been developed. In the specific case examined, calmodulin has been biotinylated, allowed to bind with appropriate proteins, and the bound calmodulin detected with an avidin linked peroxidase.

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Objectives: Calmodulin-dependent binding proteins were observed to have a high capacity to accept methyl groups transferred by the enzyme protein carboxyl-O-methyltransferase (PCM). This study examined several homogeneous calmodulin binding proteins, namely, calcineurin, a calmodulin-dependent phosphatase, Ca^{+2}-calmodulin stimulated phosphodiesterase, and purified Ca^{+2}-calmodulin dependent protein kinase.

Another objective was to specifically determine the proteins to which calmodulin binds.

Methods: Methyl acceptor capacity and molar stoichiometry was estimated by incubating the purified PCM with proteins, [^{3}H]methyl-S-adenosylmethionine, followed by precipitation and analysis of methyl group incorporation using 1) direct counting of the purified precipitate and 2) acidic SDS-gel electrophoresis. The calmodulin binding proteins phosphodiesterase and calcineurin were kindly provided by Dr. R. Kincaid, NHLBI.

In addition, substrate dependency analysis, time course, and methylester stability studies were conducted on the purified preparations, using direct analysis of methanol formed after acid precipitation followed by basic hydrolysis.

Enzyme activities for both phosphodiesterase (using cAMP as a substrate) and calcineurin (using para-nitrophenylphosphate as a substrate) were determined after carboxylmethylation.

Biotinylation of Calmodulin: Homogenous brain calmodulin (4.0 ml; 1.2 mg/ml) was dialysed overnight at 4°C against 0.1 M phosphate buffer, pH 7.4. Biotinyl-epsilon-amino-caproic acid N-hydroxysuccinimide ester was dissolved in N,N, dimethylformamide (3.2 mg/100 µl) and added to the calmodulin solution at a final concentration of 1 mM. Incubations were for 2 hr at 4° with constant stirring, followed by exhaustive dialysis. The molar ratio between the biotinylating agent and calmodulin was kept at 14:1, which is twice the number of lysine sites on calmodulin.

Major Findings: To summarize the results reported in the previous report, it was found that a number of calmodulin-binding proteins were exellent substrates for protein carboxy-O-methyltransferase (PCM). The calcium calmodulin dependent phosphodiesterase could be significantly methylated and once methylated the enzyme was stimulated to a smaller degree by the calcium-calmodulin. Basal activity, however, was unaffected. Examination of the calcium calmodulin protein kinase also showed that it was an excellant methyl group acceptor and that the enzyme once subjected to methylation could no longer respond to calcium calmodulin. Since this protein kinase from brain has a strong dependency upon calcium and calmodulin, the effects of methylation are highly significant. With both these enzymes, however, the degree of methylation was less than stoichiometric. A third calcium calmodulin stimulated enzyme is calcineurin. This enzyme exhibits a protein phosphatase activity and binds calmodulin very tightly. We were able to demonstrate that calcineurin could accept up to two molecules of methyl groups per molecule of calcineurin subunit. Like the other enzymes, once carboxymethylated calcineurin no longer responded to the stimulation by calcium and calmodulin. Basal activity was, however, not affected.

In this work, it was demonstrated that three important calcium calmodulin binding proteins could accept methyl groups and that their activity could be regulated by this methylation. Whether this methylation represents a significant in vivo regulatory role remains to be determined.

In an attempt to determine the cellular localization of calmodulin binding proteins in brain, a new method to detect calmodulin binding proteins was developed. This method is based on the coupling of an activated derivative of biotin to calmodulin. The biotinated calmodulin is then allowed to interact with calmodulin binding proteins on tissue slices or on membrane fragments. Once the excess unbound biotinylated calmodulin is removed, the calmodulin bound to specific proteins can be detected using an avidin coupled peroxidase reaction. Using this method, the distribution of calmodulin binding proteins in the central nervous system has been examined.

Significance to Biomedical Research: The function of carboxylmethylation has remained enigmatic. The high stoichiometry of phosphodiesterase and calcineurin carboxylmethylation suggests that calmodulin binding proteins may be the prime substrates for PCM. In both cases, calmodulin-activated activity was inhibited, while basal activity was not affected. Thus, carboxylmethylation may modulate the activity of phosphodiesterase. More importantly, carboxylmethylation may also affect the tyrosine phosphatase activity of calcineurin.

Proposed Course of Research:

1. Physical biochemical studies will be undertaken to determine if carboxylmethylation alters the binding of calmodulin to carboxylmethylated calcineurin.

2. Calcineurin phosphatase activity will be determined after carboxylmethylation using a phosphotyrosyl protein substrate.

3. Attempts will be made to co-localize PCM and calcineurin in the CNS.

Publications

Billingsley, M.L., Kincaid, R.L. and Lovenberg, W.: Calcineurin: Stoichiometric carboxylmethylation and subsequent inactivation of calmodulin-stimulated phosphatase activity. Proc. Natl. Acad. Sci., in press, 1985.

Billingsley, M.L., Pennypacker, K.R., Littleton, C.G., Brigati, D.J., and Kincaid, R.L.: A rapid and sensitive method for detection and quantification of calcineurin and calmodulin-binding proteins using biotinylated calmodulin. Proc. Natl. Acad. Sci., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03537-03 HE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunohistochemical Localization of Protein-O-Carboxylmethyltransferase in Brain

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Melvin L. Billingsley	Staff Fellow	HE NHLBI
Other:	Donald Kuhn	Pharmacologist	HE NHLBI
	Ulrike Berresheim	Visiting Scientist	HE NHLBI
	Cary D. Balaban	Asst. Proff. Dept. Anatomy	Hershey Med. Ctr. Hershey, PA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Both biochemical and immunohistochemical techniques were used to demonstrate the presence of protein-O-carboxylmethyltransferase (PCM; E.C. 2.1.1.24) in the CNS. The highest levels of immunoreactivity were detected in cortex, hippocampus, corpus striatum, thalamus, and the amygdala. Other brain areas exhibited lower amounts of immunoreactive PCM. Most of the immunoreactive cells were neuronal with prominent labelling detected in both the cell body and the axonal region. Biochemical analysis of PCM activity correlated with the immunohistochemical localization. Methyl acceptor substrates for the enzyme were also high in regions rich in PCM. Western immunoblot analysis of bovine brain, rat brain and human erythrocyte forms of the enzyme showed the antisera generated against bovine brain PCM cross-reacted with human and rat forms of the enzyme, suggesting structural homology. These results suggest that PCM has an unique neuronal pattern of distribution, and that carboxylmethylation of proteins may be of functional significance in the nervous system. The levels of PCM enyme activity and immunoreactivity were sufficiently high in the locus coeruleus (LC) and substantia nigra (SN) to warrant comparison with tyrosine hydroxylase (TH) immunoreactivity. Western blot analysis confirmed that the antibodies were specific and did not cross react with the other antigen of the comparison. Both anti-PCM and anti-TH heavily labelled cells within the LC and SN and it appears that the antigens are colocalized within the same cells in these brain areas.

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Objectives: PCM transfers methyl groups from S-adenosylmethionine (AdoMet) to free glutamic and/or aspartic acid residues on methyl acceptor protein (MAP) substrates. PCM activity is highest in brain, and has been postulated to participate in neuronal signal transduction. However, the distribution of PCM in brain has not been examined using histochemical methods. We have used an antibody generated against purified bovine brain PCM to show that PCM is localized in neurons throughout the brain, with highest levels found in the hippocampus, the striatum, the cortex and the thalamus. Furthermore, relatively high levels of PCM enzyme reactivity were observed in the LC and SN, two brain areas important in the catecholamine (CA) neuronal system. Therefore, these studies were extended in order to compare the cellular localization of PCM with that of TH.

Methods: Antisera was generated in rabbits, using purified bovine brain PCM as an antigen. Antiserum was collected, and an immunoglobulin fraction was partially purified by fractionation with 30% ammonium sulfate. Antisera in dilutions of 1:1000-1:5000 was incubated with 100 μsections of formalin-fixed rat brain, using phosphate-buffered saline with 20% normal goat serum as a diluent. After incubation, sections were washed, incubated with a biotinylated goat-antirabbit IgG, and again washed with phosphate-buffered saline. An avidin-peroxidase complex was added, and the multiple antibody complex was visualized using diaminobenzidine as a substrate. In some experiments, PCM immunoreactivity was visualized using FITC-conjugated goat-antirabbit IgG, followed by viewing on a fluorescence microscope. Peroxidase-stained sections were mounted, cleared and dehydrated through a graded series of ethanol/xylene and viewed using bright-field illumination.

For western immunoblot analysis, PCM from bovine brain, rat brain, and human erythrocytes or TH from PC12 cells were electrophoresed in the prescence of sodium dodecyl sulfate, and the proteins were then transferred to nitrocellulose paper. The excess protein binding sites were blocked using bovine serum albumin and hemoglobin, and antisera to PCM or TH were incubated with the blot (1:500). Immunoglobulin binding to PCM and TH was detected using either the avidin/ biotin/peroxidase system described earlier, or with goat-antirabbit-IgG coupled directly to horseradish peroxidase.

Biochemical analysis of PCM activity and MAP capacity was carried out using the alkali-induced release of ^{3}H-methanol from carboxylmethylesters as an index of PCM activity. When MAP capacity was examined, calcineurin, a calmodulin-dependent phosphatase, was used as a substrate.

Major Findings: PCM was localized in neurons throughout the rat brain. The hippocampus was prominently labelled, with pyramidal and granule cells in all regions showing immunoreactivity. The cortex also showed extensive immunoreactivity, and neurons in all layers (I-VI) appeared labelled. The striatum and thalamus also had substantial immunoreactive PCM present, while areas of the brainstem and cerebellum were only sparsely labelled. Two areas with especially dense PCM immunoreactivity were the LC and SN.

Western immunoblot analysis indicated that antisera generated against bovine brain PCM could label both human erythrocyte and rat brain forms of the enzyme. All forms of the enzyme exhibit similar kinetic constants, amino acid

compositions, and mobility on SDS-polyacrylamide gels. Thus, there is a strong likelihood that PCM is conserved between species. Similar analysis of antisera directed against TH revealed that anti-TH recognized just one band on a western blot of rat brain proteins and this band corresponded precisely in molecular weight to that of native TH. Anti-PCM did not recognize TH nor did anti-TH recognize PCM.

Biochemical analysis of rat brain regions revealed that regions with high PCM immunoreactivity also expressed high enzyme activity. MAP capacity was similarly distributed, again suggesting that the immunochemical localization corresponded with biochemical measures of protein carboxylmethylation. Finally, numerous cells in the LC and SN contained both PCM and TH, suggesting a functional interaction between PCM and CA production.

Significance to Biomedical Research: This study marks the first time that PCM has been localized in regions of brain. The unique neuronal distribution correlated with biochemical activities, and suggests a functional role for PCM in neurons. The high degree of immune cross-reactivity, along with other biochemical similarities suggests that this enzyme is conserved among mammals. This study provides a strong basis for examination of the role of PCM in CNS activity. One strong possibility lies within the LC or SN. Both areas are densely stained by anti-PCM and anti-TH and several cells contained both the antigens suggesting the PCM may indeed influence CA production in brain.

Proposed Course of Project:

1. Neurochemical lesions of specific brain regions (6-hydroxydopamine; kainic acid) to produce specific anatomic changes will be performed and correlated with alterations in tissue PCM immunoreactivity.

2. Other antisera for neurotransmitters could be used to see if PCM is co-localized with any specific transmitter system.

3. The hippocampus will be used as a model brain region to study changes in PCM activity and content as a result of various pharmacologic manipulations.

4. Pure TH will be tested as a substrate for PCM and the effects of PCM on TH activity will be examined as well.

Publications:

Billingsley, M.L., Kim, S., and Kuhn, D.M.: Immunohistochemical localization of protein-O-carboxylmethyltransferase in rat brain neurons. Neuroscience 15: 159-171, 1985.

Billingsley, M.L., and Balaban, C.D.: Protein-O-carboxylmethyltransferase in the rat brain: high regional levels in the substantia nigra, locus coeruleus, and paraventricular nucleus. Brain Research, 1985, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03538-03 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Basic and Clinical Studies with Tetrahydrobiopterin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Walter Lovenberg	Laboratory Chief	HE NHLBI
Others:	Robert Levine	PRAT Fellow	IRP NIMH
	David Pickar	Psychiatrist	BP
	Peter LeWitt	Assistant Professor	Wayne State Univ.

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	0.2	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Work has been undertaken to investigate the role of tetrahydrobiopterin (THB) in the synthesis of specific neurotransmitter substances. THB is the specific electron donor for several aromatic amino acid hydroxylases and is key for the synthesis of biogenic amines. An important aspect of our work during the past year has been an attempt to find an inhibitor of the synthesis of THB. Studies with PC12 cells suggest that N-acetyl serotonin may slow the synthesis of this important compound. Preliminary studies, however, indicate that while the levels of THB may be reduced in cells treated with N-acetyl serotonin little change occurs in the synthesis of catecholamines within these cells.

In previous years we had attempted to examine the effect of administration of THB to man to determine whether it would enhance biogenic amine synthesis and whether such enhancement would be of benefit in diseases such as Parkinson's disease, dystonia, and endogenous depression. It would appear that the neurological symptoms in certain patients with dystonia are significantly improved by treatment with THB. Little clinical response was seen in Parkinson's disease or endogenous depression. However, the poor clinical response may be due to the fact that THB appeared to penetrate the blood brain barrier very poorly.

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Objectives: The objective of our basic studies were as follows:

Studies were directed at further understanding the relationship between tyrosine hydroxyation and the availability of the hydroxylase cofactor. By examination of PC12 cells for tyrosine hydroxylase and cofactor under conditions which stimulate levels of tyrosine hydroxylase it should be possible to determine whether tyrosine hydroxylase and tetrahydrobiopterin are coordinately controlled.

The objective of another study was to further understand the biosynthetic mechanism by which GTP is converted tetrahydrobiopterin. The work was specifically devoted to the question of whether the final step in THB synthesis is catalyzed by the enzyme dihydrofolate reductase. Pineal glands were chosen for this study because they can be easily cultured and they contain a very high amount of BH_4. In this system we could evaluate the role of dihydrofolate reductase.

We also attempted to determine the ultimate clinical efficacy of tetrahydrobiopterin in various diseases where some positive responses have been noted, such as Parkinson's disease, dystonia, and endogenous depression. It is also important to understand why the clinical responsiveness is variable in the patient population.

Methods: PC12 cells were kindly supplied by Dr. Gordon Guroff's laboratory and were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum and 5% horse serum in 10% CO_2 Dexamethasone was added to the medium in 0.01% ethanol (control medium lead similar addition of ethanol). Reduced and oxidized biopterin were analyzed by differential iodine oxidation and HPLC (Fukoshima and Nixon, Anal. Biochem. 102, 176, 1980). Tyrosine hydroxylase was monitored by a standard tritium release procedure.

Major Findings: Treatment of cultures of PC12 cells with varying concentrations of N-acetyl serotonin lead to significant reductions in the THB content of these cells. It appeared, however, that at least 10^{-4} M N-acetyl serotonin was required to exert a significant effect. Measurement of norepinephrine in cells so treated failed to reveal a significant loss of catecholamines. While the results of these experiments do not lead to any definitive conclusions, it would appear that this system deserves further exploration. Since this appears to be an example in which the content of THB within cells can be dissociated from the catecholamine biosynthetic rate.

Significance to Biomedical Research: A number of human diseases may be related to an over or under activity specific types of neurons. An understanding of the fundamental regulatory properties and ways to manipulate the activity of certain specific types of neuron will lead both to better understanding of the system and potentially new therapeutic approaches. In the current study we have pursued an earlier observation suggesting that tetrahydrobiopterin levels are low in some patients with familiar dystonia. Several patients have shown significant neurological improvements with this treatment.

Proposed Course of Project: A large clinical trial with BH_4 is planned in collaboration with NIMH to determine the merits of long-term treatment of

depressed patients. New patients with dystonia will also continue to be treated with BH_4 in collaboration with Peter LeWitt at Wayne State University and neurologist in NINCDS. The collaboration with Curtius and Niederwieser in Zurich will focus on ways of enhancing BH_4 entry into brain from the periphery by coupling BH_4 to molecules which are actively transported into brain.

Publications:

Levine, R.A., Lovenberg, W., Curtius, H.-Ch., and Niederwieser, A.: Penetration of reduced pterins into rat brain: Effect on biogenic amine synthesis. In: Pteridines and Folic Acid Derivatives, Walter de Gruyter, Berlin, New York, 1983, pp. 177-182.

LeWitt, P., Miller, L., Insel, T., Calne, D., Lovenberg, W., Levine, R. and Chase, T.: Tyrosine hydroxylase cofactor (tetrahydrobiopterin) in parkinsonism. Advances in Neurology 40: 459-462, 1984.

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Culvenor, A., Miller, L., Levine, R., and Lovenberg, W.: Effects of methotrexate on biopterin levels and synthesis, in rat cultured pineal glands. J. Neurochem. 42: 1707-1714, 1984.

Culvenor, A., Zabrenetsky, V. and Lovenberg, W.: Effects of dexamethasone on biopterin levels and the tyrosine hydroxation system in PC-12 cells. Biochem. Pharmacol. 33: 2361-2366, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03539-02 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Serotonergic Innervation of the Nucleus Tractus Solitarius.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Donald M. Kuhn	Pharmacologist	NHLBI HE
Others:	William A. Wolf	Guest Worker	NHLBI HE
	Ulrike Berresheim	Visiting Scientist	NHLBI HE

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The origin of the serotonergic (5-HT) innervation of the nucleus tractus solitarius (NTS) was studied by injecting fluorescent dyes or enzymes which are transported in a retrograde direction into the NTS. The dorsal raphe nucleus was also ablated with electrolytic lesions and the effects on 5-HT levels in the NTS and other brain areas were determined by HPLC. Finally, unilateral removal of the nodose ganglion was tested for its effects on NTS 5-HT levels. The results from these studies leave open the question of 5-HT innervation of the NTS since neither raphe lesions nor nodose ganglionectomy altered NTS 5-HT levels or its metabolism in any detectable fashion. Furthermore, fluorescent retrograde dyes could not be reliably traced from the NTS to any major 5-HT nuclei.

S7K

Objectives: The nucleus tractus solitarius (NTS) is a dorso-medial medullary nucleus and is the site where primary afferents of the baroreceptor pathway first synapse. The chemical transmitter which modulates the baroreceptor reflex at the level of the NTS is not known but the NTS contains very high levels of serotonin (5-HT). Furthermore, microinjections of 5-HT into the NTS produce transient pressor effects in rats (Wolf, Kuhn, and Lovenberg, Eur. J. Pharm. 69: 291-299, 1981). The first step in learning how a brain structure might function involves the delineation of its chemical anatomy. Therefore, the objective of these studies was to determine the site of origin of the nerve endings in the NTS which contain 5-HT.

Methods: Male Sprague-Dawley rats were anesthetized and placed in a stereotoxic apparatus. The dorsal surface of the medulla was exposed by a partial laminectomy and the atlanto-occipital membrane was carefully retracted. The dyes True Blue or Propidium iodide were injected into the NTS by visually placing a single-barrel micropipette containing either dye 1.0 mm lateral to the obex and 1.0 mm ventral to the surface of the medulla. Injections of 1.0 μl were made over a 15 min time period. In some experiments, horse radish peroxidase (HRP) was injected in the same manner.

After a survival time of 3-7 days, rats were perfused transcardially with 4% formaldehyde and serial 50 sections were subsequently cut through the mesencephalon. Immunohistochemical processing was carried out by incubating free floating sections with primary antiserum (anti-serotonin) diluted 1:500. After washing, a secondary antibody crosslinked with FITC was added to the sections. Finally, sections were mounted on slides, coverslipped, and viewed on a Leitz Dialux EB20 fluorescence microscope equipped with epi-illumination. For visualization of retrogradely transported HRP, the sections were incubated with diaminobenzidine and H_2O_2, followed by mounting, dehydration through graded ethanols and zylene, and finally coverslipping in Permount for bright field microscopy.

5-HT, tryptophan, and 5-HIAA in brain tissue was determined by HPLC with fluorescence detection.

Major Findings: Injection of dyes or HRP into the NTS were not seen to label any of the major 5-HT producing cell body groups within the mesencephalon or medulla. Similarly, HRP was not transported back to the dorsal raphe. Since the substance P (a neuroactive peptide) content of the NTS arises, in large part, from the nodose ganglia, the ganglion was removed unilaterally from rats and the effects of the surgery on NTS 5-HT was investigated. The extripated nodose ganglion was also assayed and found to contain 5-HT and 5-HIAA. However, at no time after surgery did nodose ganglionectomy alter NTS 5-HT levels. Finally, the dorsal raphe nucleus was lesioned electrolytically since it is the nucleus with the highest content of 5-HT in brain. Lesions of the raphe which dramatically lowered 5-HT levels in the striatum did not alter NTS 5-HT.

Significance to Biomedical Research and Institute Programs: These results provide strong evidence that the source of 5-HT in the NTS is not the dorsal raphe nucleus nor does it appear to be the nodose ganglion. The 5-HT may be intrinsic to the NTS, since synthesis can occur there, or 5-HT may be in non-neuronal cellular elements within the NTS complex. These results also establish

that the nodose ganglia contains considerable amounts of 5-HT. Based on the influence that 5-HT application onto the ganglion has (respiratory alterations, sleep), 5-HT may play a far wider physiological role within the nodose ganglion than previously realized. Finally, in the course of these studies, significant differences in the labelling of cells by TB and PI were noted. Coinjection of these fluorescent dyes, in equimolar concentrations, into brain areas, produced very different labelling patterns and different rates of transport. These results establish that these dyes are not taken up by all neuronal elements by the same mechanism and that the dyes are not transported at the same rate. Therefore, false positive or false negative results can be arrived at in neuroanatomical experiments if careful comparative experiments are not carried out.

Proposed Course of Project:

1. The status of 5-HT within the nodose ganglion is poorly understood so that immunocytochemical studies will be carried out with 5-HT antibodies to determine the cell type which contains 5-HT. Synthesis and degradation of 5-HT will be manipulated pharmacologically to examine whether ganglion 5-HT will respond to drugs in the same fashion as 5-HT in brain.

2. Retrograde dyes will be injected into identified neuronal pathways (e.g., nigrostriatal) and their co-transport and co-localization within identified neurons will be investigated.

3. Peripherally acting pressor and depressor agents will be administered to rats to provoke the baroreflex. The effects of the treatments on 5-HT content, metabolism, and turnover within the NTS will be investigated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03540-02 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Release of Serotonin: Evidence in Support of Cytoplasmic Release.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	William A. Wolf	Guest Worker	NHLBI HE
Other:	Donald M. Kuhn	Pharmacologist	NHLBI HE
	Moussa B.H. Youdim	Visiting Scientist	NHLBI HE

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The in vivo release of serotonin (5-HT) was investigated by studying a behavioral/neurological syndrome in rats. The syndrome is referred to as the 5-HT syndrome and is comprised of a variety of symptoms including head weaving, forepaw treading, hindlimb abduction, and tail-lashing. The syndrome was presently studied in rats which had been treated with reserpine to abolish the vesicular pool of 5-HT. Injections of the 5-HT releasing drug parachloroamphetamine (PCA) produces the entire syndrome in reserpinized rats just as it does in normal rats. Parachlorophenylalanine, which inhibits the synthesis of 5-HT, or metergoline, the 5-HT receptor antagonist, each prevent the PCA effect in reserpinized rats indicating that the 5-HT remaining after reserpinization represents a newly synthesized and releaseable cytoplasmic pool of transmitter. Increases in this cytoplasmic pool with injections of a non-selective monoamine oxidase inhibitor or with a selective inhibitor of MAO-A, but not with a selective inhibitor of MAO-B, markedly reduces the dose of PCA necessary to produce the 5-HT syndrome. Thus, increases or decreases in the cytoplasmic pool of 5-HT can increase or decrease in vivo release, respectively, induced by PCA. Furthermore, since acute injection of reserpine does not produce the 5-HT syndrome, the 5-HT liberated by the alkaloid into the cytoplasm is deaminated to 5-HIAA without release occurring. Pharmacological studies confirmed this suggestion and indicate that the levels of 5-HIAA do not reflect 5-HT neuronal activity but indicate the level of activity of monoamine oxidase.

5 of 1

Objectives: Serotonin (5-HT) is a neurotransmitter in the brain and alterations in its function are thought to underlie a diverse variety of disorders ranging from hypertension to depression. Despite the status it has achieved as a neurotransmitter, 5-HT may not always behave as other classical neurotransmitters. The one aspect of neurotransmitter function which is least understood for 5-HT is perhaps the most important one - release. Serotonin is stored in two separate pools in brain (vesicles and cytoplasm) and it has been assumed that the transmitter is released by exocytosis. However, evidence in support of the exocytotic release of 5-HT is sparce. In an effort to learn more about 5-HT release in vivo, we have studied a behavioral syndrome which has been shown to be a specific and reliable assay for 5-HT release. Furthermore, these experiments were carried out in reserpinized rats since reserpine abolishes the vesicular pool of 5-HT. Any release of 5-HT would logically occur from the cytoplasmic pool which is resistant to reserpine.

Methods: Male Sprague-Dawley rats were injected with 5.0 mg/kg reserpine 24 hr prior to testing. After test injections, rats were observed and scored for expression of the characteristic symptoms of the 5-HT syndrome (i.e., head weaving, forepaw treading, hindlimb abduction, and tail lashing). All animals were sacrificed at the appropriate times and brain areas were assayed for 5-HT as described by Wolf and Kuhn (J. Chromatog. 275: 1-9, 1983). In some experiments, rats were studied immediately after reserpine injection in order to examine the acute effects of the alkaloid on 5-HT release.

Major Findings: Reserpine causes a large reduction in 5-HT levels. Within 24 hr of reserpine, 5-HT is reduced throughout the brain and spinal cord by 90-95%. Despite this reduction in 5-HT, the 5-HT releasing drug parachloroamphetamine (PCA) could still elicit the entire 5-HT behavioral syndrome. PCPA, which inhibits 5-HT synthesis, and metergoline, a 5-HT receptor blocker, each prevented the PCA-induced syndrome. Treatment of reserpinized rats with a nonselective inhibitor of MAO increases 5-HT levels almost 10 fold. This treatment drastically reduces the dose of PCA necessary to produce the 5-HT syndrome. The selective MAO-A inhibitor clorgyline also shifts the PCA dose-response curve to the left while deprenyl, a selective MAO-B inhibitor, does not.

In the time after reserpine injection (0-4 hrs) when 5-HT levels are decreasing at their fastest rate and 5-HIAA levels are increasing at their fastest rate, rats do not display the 5-HT syndrome, indicating that very little release of 5-HT has occurred. In order to test more completely whether 5-HT is being metabolized to 5-HIAA before release occurs, rats were pretreated with an monoamine oxidase inhibitor prior to reserpine. Inhibition of MAO allowed the expression of the complete syndrome once animals were reserpinized. To rule out further that release was occurring, rats were pretreated with fluoxetine in order to prevent any re-uptake of the released 5-HT. However, these animals did not display the syndrome after reserpine.

Significance to Biomedical Research and Institute Programs: These results establish that the release of 5-HT can occur even when the vesicular stores of this transmitter have been abolished. Increases or decreases in the remaining cytoplasmic pool of 5-HT produces a corresponding increase or decrease in release.

These data minimize a role for exocytosis in mediating 5-HT release and suggest that the releasable or functional pool of 5-HT is cytoplasmic. These results have a profound effect on how 5-HT is viewed. Furthermore, the use of 5-HIAA as an index of the status of the 5-HT neuronal system has been seriously questioned. 5-HIAA is the eventual product of MAO action on 5-HT and its levels simply and accurately reflect the action of this enzyme cascade, not release or the levels of neuronal activity as is most often concluded. These data must provoke a re-evaluation of the status of 5-HT in the pathophysiology of hypertension and depression since it was the measurement of 5-HIAA which formed the early basis for invoking a role for 5-HT in such disorders.

Proposed Course of Project: Experiments will be carried out in an attempt to selectively manipulate one pool of 5-HT without altering the other. For example, rats can be injected with a submaximal dose of the 5-HT synthesis inhibitor parachlorophenylalanine (PCPA) to lower 5-HT concentrations. Drugs which alter 5-HT (e.g., tryptophan, MAO inhibitors, uptake blockers) will then be given to see if 5-HT responds as it would without PCPA.

Attempts will be made to separately determine vesicle vs cytoplasmic 5-HT levels. Perhaps then release can be more predictably manipulated by agents which selectively influence one transmitter pool or the other, or the equilibrium between the various pools.

Publications:

Kuhn, D.M., Murphy, D.L., and Youdim, M.B.H.: Physiological and clinical aspects of monoamine oxidase. In Mondovi, B. (Ed.): Structure and Function of Amine Oxidases. New York, CRC Press, 1985, in press.

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Kuhn, D.M., Wolf, W.A., and Youdim, M.B.H.: The regulation of 5-HT neurotransmission by MAO: Termination or prevention of 5-HT action? J. Pharm. Pharmacol. 36: 46, 1984.

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Wolf, W.A., Youdim, M.B.H., and Kuhn, D.M.: Does brain 5-HIAA indicate serotonin release on monoamine oxidase activity. Eur. J. Pharmacol. 109: 381-387, 1985.

Kuhn, D.M., Wolf, W.A., and Youdim, M.B.H.: Serotonin neurochemistry revisited: A new look at some old axioms. Neurochem. Int., 1985, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03541-02

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Vascular Smooth Muscle Cells in Culture

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Toru Nabika	Visiting Fellow	HE NHLBI
Other:	Paul Velletri	Staff Fellow	HE NHLBI
	Walter Lovenberg	Laboratory Chief	HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.8	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In these studies we have explored the response of smooth muscle cells isolated from the aortas of rats and grown in culture to various receptor agonists. Beta-agonists strongly stimulate the accumulation of cAMP but have little effect on intracellular free calcium. In contrast, angiotensin II and arginine vasopressin cause rapid and marked increases in intracellular free calcium but have little effect on the accumulation of cAMP. In the case of arginine vasopressin, the enhancement of intracellular calcium was dependent entirely upon extracellular calcium. In contrast, angiotensin II appeared to stimulate both uptake of extracellular calcium and release of intracellular calcium. An examination of phosphotidylinositol turnover revealed that this biochemical event was closely correlated with the accumulation of intracellular calcium, both events were blocked by the appropriate antagonist. Additional studies have now shown that angiotensin II, which has little effect on the accumulation of cAMP, strongly potentiates the weak accumulation of cAMP in response to vasoactive intestinal polypeptide. Such a powerful synergism appears to offer an opportunity for further understanding of how neuropeptides interact at their cellular receptor sites.

5-84

GPO 914-918

Objectives: Our research has attempted to clarify mechanisms by which vascular smooth muscle cells (SMC) regulate the levels of intracellular second messengers, and to understand the relationship between these messengers and the physiological functions of the cells. We have attempted to delineate how SMC control the turnover of cyclic nucleotides (in particular cyclic AMP) and intracellular free calcium (Ca^{++}) as a response to a number of well-characterized vasoactive substances. Furthermore, we have studied whether there exist differences in the regulation of cAMP or Ca^{++} levels when values in SMC derived from normotensive Wistar-Kyoto (WKY) rats and their spontaneously hypertensive (SHR) counterparts were compared.

These studies have been conducted in cell culture from cells explanted from age-matched WKY and SHR. The use of cultured vascular SMC allows us to observe biochemical and pharmacological events in cells that have been isolated from the potential factors to which SMC might be exposed in vivo, such as blood pressure and neuronal or humoral influences. Hence, phenomena that are observed in culture may be thought to be the result of intrinsic cellular factors and not the result of physiological events unrelated to the biology of SMC.

Methods:

(i) SMC Culture: SMC were derived from thoracic aortas of WKY, SHR and stroke-prone SHR (SHRSP) by the explantation method described by Ross (JCB 50: 172, 1971). Cultured SMC were grown in culture flasks and the medium changed every three days. Cells were transferred to new flasks at confluence (every 7-10 days). In cell experiments, cells of 3-12 passages were used.

(ii) cAMP Determinations: Confluent cell cultures (approximately 1×10^6 cells/dish) were incubated at $37^{\circ}C$ in the presence of a variety of vasoactive substances for different periods of time (see results). At the end of the incubation periods, intracellular cAMP was extracted into perchloric acid (PCA) at $4^{\circ}C$. Cyclic AMP in the PCA extracts (supernatant fraction) was quantified with the use of a radioimmuoassay kit obtained from New England Nuclear. The residual pellet was used as a source of protein, which was determined by the method of Bradford (Anal. Biochem. 72: 248, 1976). The pellet was solubilized in 1N NaOH prior to assay.

(iii) Measurement of Intracellular Free Calcium: The fluorescent calcium indicator quin II was used for the assay of free cytoplasmic calcium in smooth muscle cells as described previously for other types of cells in culture. Smooth muscle cells were incubated in serum free medium for 24 hours prior to the loading of the cells with the quin II methylester. Following loading, the excess quin II was removed by washing the cells. Such cells could then be used for measuring the responses to various agonists.

(iv) Phosphoinositide Breakdown: Phosphoinositide breakdown was determined by a previously established technique. Cells were loaded with radioactive inositol and allowed to incubate for approximately 16 hours in serum-free medium. Once the cells were loaded with the radioactive inositol, the breakdown of phosphoinositides could be measured by separation of the radioactive products that occur in response to a specific stimulus.

Major Findings: Angiotensin II at a concentration of 100 nanomolar proved to be a potent stimulus to the uptake of calcium and release of intracellular calcium. The intracellular free calcium levels rose sharply within seconds and slowly returned toward baseline over a course of several minutes. Arginine vasopressin was somewhat less potent in this calcium response, however, a concentration of 1 M arginine vasopressin also caused a very rapid and pronounced increase. Serotonin and prostaglandin F_2 each caused a very slow and slight rise in intracellular free calcium. On the other hand, neither alpha- nor beta-agonists had any significant effect. An antagonist to the angiotensin receptor totally blocked this response to angiotensin II whereas arginine vasopressin antagonist totally blocked the response of the cells to AVP. However, there were no cross effects of these two antagonists. Examination of dose response curves showed that the enhancement of intracellular free calcium and the stimulation of phosphoinositide breakdown where closely coupled.

In another series of studies, we examined the effects of angiotensin II and vasoactive intestinal peptide (VIP) on the accumulation of cAMP by smooth muscle cells. Of interest was the finding that VIP caused a 5 to 10-fold increase in the content of cyclic AMP. On the other hand, angiotensin II had little effect on the cellular cAMP content, however, addition of angiotensin II to cells being stimulated by VIP resulted in a 50-to 100-fold increase in cAMP levels. The synergistic effect of these two peptides is of considerable interest and may reflect the previously stated sharp rise in intracellular free calcium due to the angiotensin II.

Significance to Biomedical Research and Institute Programs: The studies described above have allowed us to establish a useful model system for the study of the pathogenesis of hypertension. The culturing of vascular SMC allows us to study indirectly the abnormalities in contractility that are the final common denominators of high blood pressure. Our ability to culture SMC and to study the regulation of second messengers involved in contractility will allow us to study the cell type (i.e., SMC) most responsible for the elevated blood pressure in hypertension. SMC can now be studied when isolated from whole animal factors such as blood pressure and neuronal or humoral influences. The goals of our research are in keeping with the general objectives of the Hypertension-Endocrine Branch and will allow us eventually to study numerous aspects of the cell biology of normo- and hypertensive SMC.

Our pilot studies have demonstrated the cAMP can accumulate following β-adrenergic stimulation and that this response is primarily associated with one vascular cell type (SMC), and not the pervasive fibroblasts. Beta-agonists are well known to cause vascular relaxation of SMC. Our pilot studies have also demonstrated that K^+ and angiotensin II cause an enhanced accumulation of Ca^{++} in SMC, a phenomenon associated with vasoconstriction. To date, no differences in the responsiveness of SMC derived from WKY, SHR or SHRSP have been observed with any parameter assayed.

Little is understood about the regulation of vascular contractility in either normal homeostatic or pathological conditions. Related to mechanisms of contractility are: (1) receptor-binding of vasoactive substances to the plasmalemma of SMC; (2) membrane transduction mechanisms; (3) the generation of intracellular second messengers, such as cAMP; (4) increases in the membrane's

permeability to ions, such as Ca^{++}; and (5) a combination of physical and biochemical events resulting in muscular contraction. We have chosen to commence our studies on the cell biology of SMC derived from normal and hypertensive animals by delineating control mechanisms for the regulation of two second messengers -- cAMP and Ca^{++}. Both have been implicated in playing a significant role in the pathogenesis of abnormalities of contractility in SMC of hypertensive individuals.

The heterogeneity of vascular tissue (i.e., endothelium, SMC, fibroblasts, etc.) has made certain conclusions with regard to SMC in intact tissue difficult to interpret in the past. Our observations that SMC appear to accumulate cAMP more extensively than the pervasive fibroblasts suggest that studying an individual cell type may lead to novel insights on the regulation of second messengers in SMC.

Proposed Course of Project: Within the next year, we anticipate studying in more detail the role of cAMP and Ca^{++} in vascular smooth muscle physiology and pathology. We will attempt to relate the cellular regulation of free cAMP and Ca^{++} levels to the pathogenesis of hypertension by comparing the cAMP and Ca^{++} regulatory systems of SMC from WKY and SHR. In particular, we anticipate studying how vasoactive substances modulate the production of cAMP that results from β-stimulation and exploring mechanisms by which free cytosolic Ca^{++} levels are controlled by vasopressor agents such as angiotensin II. These latter studies will employ novel and sensitive techniques for the measurement of free Ca^{++} by using the fluorescent Ca^{++} indicator, quin 2. These studies are being undertaken with the collaboration of Dr. Michael Beavan of the Laboratory of Chemical Pharmacology.

Publications:

Nabika, T., Velletri, P.A., Igawa, T., Yamori, Y. and Lovenberg, W.: Comparison of cyclic AMP accumulation and morphological changes induced by β-adrenergic stimulation of cultured vascular smooth muscle cells and fibroblasts. Blood Vessels 22: 47-56, 1985.

Nabika, T., Velletri, P.A., Lovenberg, W., and Beaven, M.A.: Increase in cytosolic calcium and phosphoinositide metabolism induced by angiotensin II and arg-vasopressin in vascular smooth muscle cells. J. Biol. Chem. 260: 4661-4670, 1985.

Nabika, T., Nara, Y., Yamori, Y., Lovenberg, W., and Endo, J.: Angiotensin II and phorbol ester enhance isoproterenol- and vasoactive intestinal peptide (VIP)-induced cyclic AMP accumulation in vascular smooth muscle cells. Biochem. Biophys. Res. Commun., in press.

Nabika, T., Velletri, P.A., Beaven, M.A., Endo, J., and Lovenberg, W.: Vasopressin-induced $[Ca^{2+}]_i$ increase was enhanced in cultured vascular smooth muscle cells from spontaneously hypertensive rats. Life Science, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03542-02 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Phosphatidylinositol Pathway in Cultured Aortic Smooth Muscle Cells.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen Buck	PRAT Fellow	HE NHLBI
Other:	Toru Nabika	Visiting Fellow	HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project was terminated and investigation of agonist- and depolarization-induced PI turnover and Ca++ influx in cultured WKY and SHR aortic smooth muscle cells was continued by Dr. Nabika as Project #Z01-HL-03541-01 HE.

SH

Publications:

Buck, S.H., Nabika, T., and Lovenberg, W.: Mn^{++}-stimulated ^{3}H-inositol incorporation in cultured aortic smooth muscle cells: Deficiency in cells from spontaneously hypertensive rats. Clin. Exp. Hyperten., in press.

Nabika, T., Velletri, P.A., Buck, S.H., Beaven, M.A., Lovenberg, W., Yamori, Y., and Endo, J.: Regulation of cytosolic free Ca^{++} concentrations and phosphoinositide metabolism in aortic smooth muscle cells from SHR and WKY. Abst. 5th Internat. Symp. on SHR and Related Studies, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03543-02 HE

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Characterization of the Multiple Types of Tachykinin Receptors.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen Buck	PRAT Fellow	HE NHLBI
Others:	Elizabeth Burcher	Visiting Scientist	ET NINCDS
	Clifford W. Shults	Medical Staff	ET NINCDS
	Thomas L. O'Donohue	Unit Head	ET NINCDS

COOPERATING UNITS *(if any)*: Neuroendocrinology Unit, Experimental Therapeutics Branch, NINCDS

LAB/BRANCH: Hypertension-Endocrine

SECTION: Biochemical Pharmacology

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	1.0	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The novel pharmacology of K-type tachykinin binding sites has been identified in several peripheral tissues. Substance K may be the mammalian endogenous ligand for this receptor whereas substance P and neuromedin K may be the endogenous ligands for P-type and E-type sites, respectively. Although all three sites are regulated identically by ions and guanine nucleotides, the distinct anatomical localization of each type of site in various tissues indicates that each is a distinct type of tachykinin peptide receptor.

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Objectives: In order to distinguish among multiple types of tachykinin peptide receptors, the pharmacological characterization and autoradiographic distribution of binding sites for radioiodinated tachykinins have been investigated.

Methods: For membrane binding studies, a standard filtration assay for peptides has been adapted for use with the various tachykinins. For autoradiography, methodology developed for ^{125}I-Bolton-Hunter-substance P (BHSP) has been adapted for use with labeled substance K (BHSK), eledoisin (BHE), and neuromedin K (BHNK). Specific binding is defined as the difference in binding in the absence and presence of 1 μM unlabeled peptide.

Major Findings: In the CNS, BHSK, BHE, and BHNK all have a similar autoradiographic distribution that is distinct from that for BHSP. BHSP binding sites are more widespread and in the brainstem BHSP labels both sensory and motor nuclei whereas the other three tachykinins label only sensory nuclei. In the gastrointestinal tract, binding sites for both BHSK and BHSP were seen in circular muscle of the rat stomach, small intestine, and colon and in circular and longitudinal muscle of the guinea-pig small intestine and colon. Binding sites for BHSK, but not for BHSP, were seen in the muscularis mucosa of the gastric fundus and colon of the rat. Binding sites for BHSP, but not for BHSK, were seen in the mucosa of the guinea-pig colon and densely clustered over ganglia of the myenteric and submucous plexuses in rat and guinea-pig colon. Pharmacological characterization of tachykinin binding in urinary bladder revealed that the hamster bladder contains a high number of K-sites and only a few P-sites while guinea-pig bladder contains primarily P-sites. The rat bladder contains a high number of P- and K-sites whereas the mouse bladder contain smaller numbers of both of these types of binding sites. The only peripheral tissue in which we have identified probable E-sites is longitudinal smooth muscle of the guinea-pig small intestine. This tissue contains all three types of tachykinin receptors - P, E, and K.

Significance to Biomedical Research and Institute Programs: Our results provide further evidence for the existence of multiple types of tachykinin receptors. Of particular importance is the finding that substance P and substance K, two coexisting related peptides, each is the endogenous ligand for a distinct receptor - substance P for P-receptors and substance K for K-receptors. Since these two types of receptors don't always coexist, this may mean that the two peptides are processed differently in some neuronal cells or that distinct substance K-containing neurons exist. Substance K may have as yet unknown important physiological and pathological functions in the CNS and periphery.

Proposed Course of Project: Termination

Publications:

Burcher, E., Shults, C.W., Buck, S.H., Chase, T.N., and O'Donohue, T.L.: Autoradiographic distribution of substance K binding sites in rat gastrointestinal tract: A comparison with substance P. Eur. J. Pharmacol. 102: 561-562, 1984.

Buck, S.H., Burcher, E., Shults, C.W., Lovenberg, W., and O'Donohue, T.L.: Novel pharmacology of substance K binding sites: A third type of tachykinin receptor. Science 226: 987-989, 1984.

Buck, S.H., and Burcher, E.: The tachykinins: A family of peptides with a brood of receptors. Trends Pharmacol. Sci. Review, in press.

Shults, C.W., Buck, S.H., Burcher, E., Chase, T.N., and O'Donohue, T.L.: Distinct binding sites for substance P and neurokinin A (substance K): Cotransmitters in rat brain. Peptides, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03544-01 HE

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Purification and Characterization of Tyrosine Hydroxylase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Donald M. Kuhn	Pharmacologist	HE	NHLBI
Others:	Eleanor Bruckwick	Chemist	HE	NHLBI
	Melvin Billingsley	Staff Fellow	HE	NHLBI
	James C. Osborne, Jr.	Senior Investigator	MD	NHLBI
	Ulrike Berresheim	Visiting Scientist	HE	NHLBI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Hypertension-Endocrine

SECTION: Biochemical Pharmacology

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Tyrosine hydroxylase (TH) has been purified to homogeneity from cultured PC12 cells. The enzyme has a subunit molecular weight of 58,800. Two dimensional electrophoresis of the purified enzyme revealled three isozymic forms differing in charge (pI between 5.3-5.6) and which were positively identified by Western immunoblots. Amino acid analysis indicated a high content of hydrophobic amino acids which was confirmed by the observation that the enzyme also binds tightly to phenylsepharose, a hydrophobic matrix. Antibodies against PC12 TH were raised in rabbits and the antibody was highly specific. Blots of crude protein preparations from brain adrenal chromaffin cells, rat striatum, and cultured PC12 cells onto nitrocellulose paper revealed that the antibody recognized a single protein of Mr 58,800 in each tissue which corresponds to the Mr of purified TH. The anti-TH also immunoprecipitated TH enzyme activity from each tissue but interaction of the antibody with TH neither activated nor inhibited enzyme activity as such. The anti-TH does not cross react with other monooxygenase enzymes including tryptophan hydroxylase and phenylalanine hydroxylase. The purified enzyme is a good substrate for various protein kinase enzymes. The enzyme shows little activity toward tryptophan as a substrate and expressed no phenylalanine hydroxylase activity whatsoever.

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Objectives: Tyrosine hydroxylase is the initial and rate limiting enzyme in the synthesis of the catecholamines (CA) dopamine and norepinephrine. Altered function of the CA neuronal systems or changes in CA secretion have been implicated in the pathophysiology of a variety of diseases ranging from hypertension to depression and schizophrenia. Furthermore, the CA are thought to be neurotransmitters with an important and integral role in a large number of normal physiological processes. The ultimate understanding of dysfunction can only arise after an understanding of normal function has been achieved and for those pathological and normal conditions wherein CA are thought to contribute, in one way or another, the rate limiting synthetic step is perhaps the best target for study. TH is known to be a very unstable enzyme and only one successful characterization of the native enzyme has been published. Therefore, the objective of this project was to purify TH from cultured PC12, characterize the protein, and produce antibodies for immunoprecipitaton and immunocytochemical studies.

Methods: PC12 were grown in culture and harvested once weekly after passage of the line. Sedimented cells were sonicated and TH was purified from the supernatant of a 105,000 x g centrifugation step. TH activity was fractionated between 30-42% ammonium sulfate and then was successively chromatographed on Sepharose 6B, DE52, and heparin-sepharose. The eluate from the heparin sepharose affinity matrix showed a single band upon SDS gel electrophoresis. The soluble protein was prepared for injection in rabbits by emulsification in Freund's adjuvant. Rabbits were innoculated with 75 µg and boosted with 40 µg at two week intervals. Serum was tested for titer by immunoprecipitation of TH activity, by Ouchterlony immunodiffusion, and by immunocytochemistry.

Major Findings: TH could be purified over 300 fold from PC12 cells to a final specific activity of 230 nmol/mg/min. The subunit molecular weight was determined to be 58,800 by SDS-polyacrylamide gel electrophoresis. Amino acid analysis indicated a relatively high content of phenylalanine and valine residues in the enzyme. The isolectric point of the enzyme fell within the range of pH 5.3-5.6 with three forms stained at the same Mr. The three isozymes were positively identified as TH by Western immunoblotting procedures. The enzyme did not depend on ferrous ion for activity and its pH optimum was between pH 6.5-7.0. The enzyme was quite specific with regard to amino acid substrate, demonstrating little if any tryptophan- or phenylalanine hydroxylating activity. Using cell free extracts from PC12 as a source of protein kinase, TH was found to be a good substrate for both cAMP- and phospholipid-dependent protein kinases.

The enzyme was used to generate polyclonal antibodies and titer appeared in the serum after 4 injections. Incubation of antiserum in Ouchterlony immunodiffusion plates vs pure TH, or PC12 cell extract revealled a single precipitin line. Western blots of crude protein preparations from brain, PC12 cells, or cultured adrenal chromaffin cells indicated that the antibody recognized a single protein from each source and their Mr corresponded to the Mr of the TH subunit. Anti-TH did not cross react with either tryptophan hydroxylase or phenylalanine hydroxylase.

Significance to Biomedical Research and Institute Programs: Careful studies on the physical properties of TH and its catalytic properties can now be undertaken with the successful purification of the enzyme from PC12 cells. Similarly, the antibody has proved extremely useful in immunoprecipitation and immunocyto

chemical experiments. The cross reactivity of the anti-TH with TH from a wide variety of tissue along with the very low cross reactivity with other monooxygenases indicates that the antiserum will enable a large number of neuroanatomical experiments not before possible. Studies on the mechanism of action of TH will increase the understanding of how CA synthesis is regulated in neurons and will allow a new approach to assessing the role of CA in such disorders as hypertension.

Proposed Course of Project:

1. Determine metal content of native enzyme.

2. Determine phosphate content of native enzyme.

3. Monitor changes in oxidation/reduction status of protein with fluorescence.

4. Develop monoclonal antibodies to pure TH.

Publications:

Billingsley, M.L., Balaban, C.D., Berresheim, U., and Kuhn, D.M.: Comparative studies on the distribution of protein-O-carboxylmethyltransferase and tyrosine hydroxylase in brain by immunocytochemistry. Neurochem. Int., 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03545-01 HE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Serotonin Uptake and Release in Synaptosomes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	William A. Wolf	Guest Worker	HE	NHLBI
Other:	Donald M. Kuhn	Pharmacologist	HE	NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertenson-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.2	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues X☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

An HPLC assay with fluorometric detection has been developed which is sensitive enough to simultaneously measure endogenous tryptophan, serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) inside of synaptosomes as well as that which has been released into the incubation medium. Using this assay, we have observed that tryptophan is rapidly taken up by synaptosomes and turned over to 5-HIAA without a concurrent release of 5-HT. Exogenous 5-HT is also rapidly taken up and, within 20-30 min, 80% of the 5-HT is deaminated. Veratridine releases both tryptophan and 5-HT from synaptosomes. Changes in the disposition of exogenous tryptophan or 5-HT can be completely accounted for by uptake or by stoichiometric changes in metabolites. This assay method should be valuable in the study of 5-HT pools and in the determination of from which pool 5-HT release occurs.

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Objectives: The objective of this project was to develop an assay which was capable of measuring tryptophan, serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in brain synaptosomal preparations. The assay must be sensitive enough to measure these substances inside of synaptosomes as well as in the medium after release. Furthermore, the use of monoamine oxidase inhibitors (MAOI), which prevent the breakdown of 5-HT by MAO, should be avoided so that conditions within synaptosomes can be maintained as close to normal as possible. The development of such an assay would then allow the release process for 5-HT to be studied in great detail.

Methods: Synaptosomes were prepared and purified from rat brain by differential centrifugation in sucrose-Ficoll gradients. Synaptosomes were harvested and incubated in a modified Krebs physiological medium at 37°C and after various manipulations, the synaptosomes were separated from the incubation medium by centrifugation. The supernatant was removed and then the pellet was homogenized and each fraction was assayed separately for tryptophan, 5-HT, and 5-HIAA by an HPLC method with fluorescence detection (Wolf and Kuhn, J. Chromatog. 275: 1-9, 1983).

Major Findings: The synaptosomal content of 5-HT (5-HT_{in}) declined by approximately 20% within 20 min at 37°C and remained stable throughout the remainder of the 60 min incubation. The levels of 5-HT in the extrasynaptosomal medium (5-HT_{out}) decrease almost 70% within 20 min at 37°C and remain stable thereafter. $Tryp_{in}$ showed a modest decline during the first 5 min of incubation then increased slightly but gradually while $tryp_{out}$ increased 10 fold throughout the 60 min incubation. The levels of 5-HIAA_{in} declined by 70% while 5-HIAA_{out} showed the reverse, increasing 10 fold over the 60 min incubation period. In contrast to results at 37°C, the levels of 5-HT, tryptophan, and 5-HIAA remained constant at 1°C both inside of synaptosomes and in the extrasynaptosomal medium for the entire 60 min incubation.

Synaptosomes actively take up exogenous tryptophan and convert it readily to 5-HT and 5-HIAA. Concentrations of tryp in excess of 5 μM "saturate" synthesis of 5-HT within synaptosomes but at the concentrations presently used (10^{-3} - 10^{-8} M), tryptophan does not cause the release of 5-HT into the medium.

Synaptosomes were "labelled" with exogenous 5-HT in order to study uptake and metabolism of the transmitter. The concentration chosen was 50 nM since this approximates the K_m for 5-HT uptake in synaptosomes and concentrations such as these are thought to preferentially label 5-HT nerve terminals. The removal of 5-HT from the medium by synaptosomes was rapid and within 10 min, all exogenous 5-HT was taken up. Of the exogenous 5-HT added (25 pmoles), more than 90% can be accounted for by stoichiometric conversion to 5-HIAA or by uptake.

Synaptosomes were depolarized with veratridine (10-50 μM) and the effects on synaptosomes were assessed. The alkaloid increased $tryp_{out}$ while decreasing $tryp_{in}$ and this effect was independent of concentration. Veratridine produced a concentration-dependent increase in 5-HT_{out} while decreasing 5-HT_{in} along the same time course. The release of 5-HT_{in} can be accounted for in the extrasynaptosomal medium as in increase in 5-HT_{out}. Finally, veratridine had little effect on 5-HIAA.

Significance to Biomedical Results and Institute Program: The assay presently developed represents the first successful method for measuring endogenous synaptosomal 5-HT without the use of an MAOI to increase 5-HT levels. The uptake, release, and metabolism of tryptophan, 5-HT, and 5-HIAA can be measured both inside of synaptosomes as well as in the medium. We have already demonstrated that tryptophan does not increase 5-HT release and our results indicate that the use of MAO inhibitors in synaptosomal preparations can lead to incorrect conclusions about release mechanisms. Finally, systematic studies on neurotransmitter release are becoming possible in relatively normal synaptosomal preparations and initial results indicate that many of the older concepts regarding transmitter release may require modification.

Proposed Course of Project: The compartmentation of 5-HT within synaptosomes will be studied in an attempt to determine how 5-HT is disposed of by synaptosomes upon synthesis or uptake. Synaptosomes will be partially depleted of 5-HT by incubation with veratridine, and the effects of this treatment on 5-HT synthesis from tryptophan and on 5-HT catabolism by MAO will be studied. Emphasis will be placed on determining the role of vesicles in 5-HT neurochemistry. A variety of drugs and conditions will also be tested to design a scheme whereby the appropriate, releaseable pool of 5-HT can be identified so that release from this pool can be elicited by the most selective means possible.

Publications:

Wolf, W.A., and Kuhn, D.M.: The uptake and release of tryptophan and serotonin: An HPLC method to study the flux of endogenous 5-hydroxyindoles through synaptosomes. J. Neurochem., 1985, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03546-01 HE

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Dopamine in the Development of Drug-Induced Neurotoxicity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Christopher J. Schmidt NRSA Fellow HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The possible involvement of dopamine in the neurotoxicity of a number of centrally acting drugs is being examined. The hypothesized role of dopamine in the selective toxicity of the parkinsonism-inducing compound 1-MPTP was investigated using mice pretreated with agents which alter endogenous dopamine synthesis in a defined manner. Treatment with the competitive inhibitor of tyrosine hydroxylase, alpha-methy-p-tyrosine, did not alter the dopaminergic neurotoxicity of 1-MPTP nor did treatment with large doses of the dopamine precursor L-DOPA.

In related experiments, the mechanism behind the requirement for dopamine synthesis in the serotonergic neurotoxicity of central stimulants such as methamphetamine was studied. Serotonergic neurons were shown to take up dopamine via their high affinity uptake system. This uptake was facilitated by ascorbic acid just as the neurotoxicity of methamphetamine requires normal levels of ascorbic acid *in vivo*. More recently we have also demonstrated that dopamine release induced by drug stimulation *in vivo* does enter serotonergic neurons where it displaces endogenous stores of serotonin.

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GPO 914-918

Objectives: The objective of these studies is to determine the conditions under which the well known potential of dopamine for oxidation to electrophilic species may become pathological in vivo. Number of investigators have suggested that dopamine may be a potent neurotoxic under some conditions and ultimately may be responsible for the development of diseases such as parkinsonism and some dementias. The recently reported identification of 6-hydroxydopamine in the brains of rats treated with methamphetamine lends additional support to these suggestions.

Methods: All in vivo experiments ultilize radioenzymatic assays and measurements of CNS monoaminergic neurotransmitters as markers of specific neuronal populations and indices of toxicity. Tyrosine hydroxylase activity, the rate limiting step in catecholamine biosynthesis, is determined by a tritium release assay developed in our laboratory. Brain concentrations of dopamine, dihydroxyphenylacetic acid, homovanillic acid, serotonin, and 5-hydroxyindoleacetic acid are measured by high performance liquid chromatography using electrochemical detection.

Tryptophan hydroxylase assays are being performed by L.A. Matsuda and Dr. J.W. Gibb of the University of Utah on a collaborative basis.

Major Findings: Most importantly, these studies have demonstrated a route through which excessive dopaminergic activity could have a direct cytotoxic effect on neighboring populations of monoaminergic cells. The accumulation of dopamine in serotonergic neurons has now been demonstrated to occur both in vitro and in vivo via the serotonin uptake carrier. In the in vivo studies still in progress, dopamine released by pharmacological manipulations (i.e., neuroleptic agents and dopamine uptake inhibitors) was shown to enter serotonergic neurons and affect serotonin stores.

In the 1-MPTP studies, our results were the first test of a popular theory of the mechanism responsible for the compound's neurotoxicity. Manipulations of dopamine stores by up to 50 percent of control had no effect on the loss of either dopamine levels or tyrosine hydroxylase activity in the mouse neostriatum. These results do not support the suggestion that 1-MPTP is neurotixic by virtue of its ability to initiate the generation of toxic oxidation products of dopamine.

Significance to Biomedical Research and Institute Programs: These studies have two lines of applicaton. On a more immediately practical level, these studies may provide an insight into the causes of some of the more common drug-induced or drug-associated neurotoxicities. Examples include the almost inevidable development of tardive dyskinesia with chronic neuroleptic treatment, the psychosis associated with amphetamine abuse and the dementias often elicited with L-DOPA treatment in parkinsonian patients. On a more basic level, the study of drug-induced neurotoxicities has lead to a greater understanding of the functions of ascorbic acid in the brain and the workings of the neurotransmitter systems in monoaminergic neurons.

Proposed Course: The intention is to continue examining the conditions under which excessive dopaminergic activity is associated with neurotoxicity; to determine the conditions leading to damage to specific neuronal populations; and

to determine the mechanism of that toxicity. Specific experiments will examine the effect of drugs known to increase dopaminergic activity such as L-DOPA, methamphetamine, and amfonelic acid on our markers of monoaminergic neuron integrity. Some of these experiments will also utilize MAO inhibitors to increase synaptic levels of dopamine and reduce its metabolism. These experiments are particularly important in view of the increasing interest in combination MAO inhibitor - L-DOPA therapy for parkinsonism and the recently resurrected idea that L-DOPA therapy may actually accelerate the progress of the disease.

We will also continue to examine the role of ascorbic acid in dopaminergic neurotransmission using in vitro release techniques. The effect of indirectly acting dopamine agonist on the inhibition of acetylcholine release from neostriatal slices will be used as a model system. The effect of ascorbic acid on this model of dopaminergic function will then be determined. Similar experiments have already demonstrated that ascorbic acid facilitates dopamine uptake by serotonergic neurons. Previous in vivo studies have suggested that ascorbic acid may also be important in the efficiency of transynaptic signaling by dopamine. These in vitro studies will delineate the mechanism responsible for this observation.

Publications

Schmidt, C.J., Bruckwick, E., and Lovenberg, W.: Lack of evidence supporting a role for dopamine in 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine neurotoxicity. Eur. J. Pharmacol (in press), 1985.

Schmidt, C.J., and Lovenberg, W.: In vitro demonstration of dopamine uptake by neostriatal serotonergic neurons of the rat. Neurosci. Letters (in press), 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03547-01 HE

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
In Vitro Studies on the Putative Dopamine Autoreceptor Agonist, B-HT 920.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Christopher J. Schmidt	NRSA Fellow	HE NHLBI
Others:	Anne Lobur	Guest Worker	HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Results from numerous in vivo studies have suggested that a new dopamine agonist, B-HT 920, may be selective for stimulation of dopamine autoreceptors. To examine this question, the inhibition of K+-stimulated [3H]dopamine and [14C]acetylcholine release was used as an in vitro measure of presynaptic and postsynaptic dopamine receptor stimulation, respectively. In contrast to the results of in vivo studies, B-HT 920 was found to be a potent agonist at both pre- and postsynaptic dopamine receptors. Both effects of B-HT 920 were stereospecific and antagonized by the D_2 dopamine antagonist, sulpiride, while the alpha antagonist, tolazoline, was without effect. The presynaptic effect of B-HT 920 was selective to dopamine neurons in that K+-stimulated [3H]serotonin release was not affected.

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Objective: These experiments were performed to determine if the dopamine agonist B-HT 920 was actually selective for the dopamine autoreceptor or if another mechanism was responsible for its apparent selectivity in vivo. The characterization of an autoreceptor agonist by such objective criteria as those utilized here would provide the first conclusive evidence that such agents could be developed.

Methods: Rat neostriatal slices dual-labeled with [^{3}H]dopamine and [^{14}C]acetylcholine were used for these experiments. Inhibition of depolarization-induced ^{3}H-dopamine release was taken as an indication of activation of presynaptic "autoreceptors" located on dopaminergic terminals. Inhibition of depolarization-induced [^{14}C]acetylcholine release was taken to indicate activation of postsynaptic dopamine receptors. Depolarization was induced by exposure to a submaximal K^{+} concentration of 20 mM. The slices were maintained in serial 5-min incubations in Kreb's Ringer bicarbonate (KRB) buffer at 37°C under 95% O_2/5% CO_2. Drugs were added directly to the KRB. After removal of the slices, LS cocktail was added directly to each of the serial fractions and releasure during that 5-min period was determined by dual label liquid scintillation counting.

Major Findings: The results of these experiments clearly showed that B-HT 920 did not selectively activate just the dopamine autoreceptor in rat neostriatal slices in vitro. Both depolarization-induced [^{3}H]dopamine release and [^{14}C]acetylcholine release were inhibited by B-HT 920 in a concentration-dependent manner. Both effects were blocked by the active neuroleptic isomer (+) butaclamol but were unaffected by the inactive isomer (-) butaclamol. According to reports on the literature, both the dopamine autoreceptors and the postsynaptic receptors inhibiting acetylcholine release are of the D_2 subtype. The effects of B-HT 920 on both dopamine and acetylcholine release were blocked by the selective D_2 antagonist, sulpiride, further supporting a nonselective action of B-HT 920. Since B-HT 920 is a potent agonist at the alpha$_2$ adrenoceptor, the effect of the alpha antagonist tolazoline was examined. Although tolazoline significantly elevated K^{+}-stimulated ^{3}H-dopamine release itself, it did not interfere with the inhibitory effect of B-HT 920 on either dopamine or acetylcholine release. The precursor of B-HT 920, B-HT 933, which is also an alpha agonist but without any reported dopamine agonist properties, was without effect in this system. Finally, the inhibitory effect of B-HT 920 on stimulated release was not a general effect of the drug since in dual label experiments, B-HT 920 had no effect on stimulated [^{3}H]serotonin release at the same time [^{14}C]acetylcholine release was depressed by the drug.

Significance to Biomedical Research and Institute Programs: The development of selective presynaptic agonists is an area of research which is gradually receiving more attention. Such highly targeted drugs would provide a number of therapeutic benefits without many of the side-effects associated with drugs of less specificity. The dopamine autoreceptor agonists have probably received the most attention in this regard to date. Currently, conditions associated with excessive dopaminergic activity, such as schizophrenia, are treated with any of a number of diverse neuroleptics (i.e., dopamine receptor antagonists). Unfortunately, a large number of these drugs eventually cause a form of drug-induced parkinsonism known as tardive dyskinesia. The syndrome is essentially permanent

and refractory to most drug therapy. The development of selective dopamine autoreceptor agonist would therefore provide an important alternative to classical neuroleptics. By acting directly at the dopamine terminal these drugs could "shut off" excessive dopamine release without the side effects of chronic postsynaptic receptor blockade. The characterization of these putative autoreceptor agonists as they are developed is obviously an important part of our continued progress toward such agents which may be of eventual therapeutic use.

Proposed Course of Project: The dopamine terminal in the neostriatum is the location of a large number of receptors including the dopamine autoreceptor. Many of these presynaptic receptors are stimulatory and many are inhibitory with respect to dopamine release; for some their exact function(s) remains to be elucidated. A great deal of what is known about these autoreceptors comes from studies of their effect on dopamine release induced by general depolarizing conditions such as high K^+ or electrical stimulation. An obvious problem with this approach is the nonphysiological nature of this stimulus and the fact that virtually all neurons are depolarized by such treatment. Because of the resultant release of a number of excitatory and inhibitory transmitters into the area of the dopamine terminal, a number of investigators have suggested that the so-called autoreceptor effect may be due to changes in the activity of local feedback circuits impinging on the dopamine terminal. To remove this complicating factor, we intent to make use of some of the excitatory heteroreceptors located on the dopamine terminal. By using nicotinic agonist to stimulate [^{3}H]dopamine release from rat striatal slices, a more specific and physiological release can be obtained. The effect of a number of putative autoreceptor agonists on this release will then be studied. These experiments are now in progress and are beginning with a characterization of nicotinic-induced [^{3}H]dopamine release from striatal slices in vitro.

Publications:

Schmidt, C.J., Lobur, A., and Lovenberg, W.: Effect of the putative dopamine autoreceptor agonist, B-HT 920, on K^+-stimulated [^{3}H]dopamine and [^{14}C]acetylcholine release. Naunyn-Schmiedeberg's Arch. Pharmacol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03548-01 HE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Synaptosomal Protein Phosphorylation by Calcium Channel Antagonists.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Phillip J. Robinson Visiting Fellow HE NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The molecular components of neuronal calcium channels have not yet been identified, however protein phosphorylation has been shown to play a role in the function of calcium channels. Two neuronal phosphoproteins, P96 and P139, were studied in rat brain synaptosomes and it was proposed that their phosphorylation may play a role in neuronal calcium channel function. Activation of calcium channels by depolarization leads to the extremely rapid dephosphorylation of these proteins. Although dephosphorylation was dependent on external calcium, it was also dependent on the mechanism of calcium entry through calcium channels, rather than simply to an increase in intracellular calcium. Thus, agents which stimulated rises in intracellular calcium did not initiate dephosphorylation. In contrast, drugs which activate (BAY K 8644) or inhibit (nifedipine, verapamil) calcium channels specifically increased the phosphorylation of P96 and P139. The data supported a proposal that these phosphoproteins may be involved in calcium channel function, either as integral proteins or regulatory proteins.

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Objectives: Two neuronal phosphoproteins, termed phosphoprotein 96 (P96) and P139, have been observed in intact synaptosomes to be regulated by depolarization. In contrast to a wide range of proteins whose phosphorylation is increased by a depolarizing stimulus, P96 and P139 are rapidly dephosphorylated on depolarization of nerve terminals. Previous findings lead to the proposal that the phosphorylation state of P96 is sensitive to the method of calcium entry into nerve terminals, through calcium channels, rather than to alterations in calcium levels itself. The aim of the study was to characterize the phosphorylation of P96 and P139 in order to examine the hypothesis that they may be involved with regulation of depolarization-dependent calcium entry.

Methods: Intact synaptosomes were prepared from either rat striatum or cerebral cortex. Synaptosomes were prelabelled in the presence of $^{32}P_i$ in order to label the intrasynaptosomal ATP pool, subjected to a brief preincubation with various drugs, and incubated in either control or depolarizing concentrations of K^+ for short times. In some experiments the synaptosomes were lysed after $^{32}P_i$ prelabelling and separated into membrane and cytosolic fractions by high speed centrifugation.

Phosphoproteins were separated by polyacrylamide gel electrophoresis and detected by autoradiography. Quantitation of the phosphorylation of individual phosphoproteins was determined by densitometry, and verified by excision of the band from the gel and liquid scintillation counting.

Major Findings: P96 and P139 have not been previously investigated by other laboratories, therefore it was important to determine some of the properties of these phosphoproteins. Both proteins became phosphorylated in intact synaptosomes, however, when the same synaptosomes were lysed and phosphorylated in the presence of exogenous ^{32}P-ATP neither protein became labelled, even in the presence of cyclic AMP, calmodulin, or phospholipids. In contrast, most synaptosomal phosphoproteins are phosphorylated under both conditions. Thus, the phosphorylation of P96 and P139 depends on a property of intact synaptosomes, such as membrane potential, that is lost upon lysis.

In order to determine which protein kinase was responsible for the phosphorylation of these proteins, intact synaptosomes were incubated with dibutyryl cyclic AMP or 8-bromo cyclic AMP. These agents act through cyclic AMP-dependent protein kinase and produced small increases in the phosphorylation of these proteins. This stimulation was also achieved in the presence of concentrations of fluphenazine that fully inhibit calmodulin-dependent protein kinase and protein kinase C. P96 was found to be largely a cytosolic protein, while P139 was largely associated with the membrane after lysis of intact synaptosomes that had been prelabelled with $^{32}P_i$.

Depolarization of synaptosomes causes a rapid dephosphorylation of these proteins, and an increased phosphorylation of many other synaptosomal proteins. P96 and P139 dephosphorylation was shown to be dependent on extracellular calcium, although not on the rise in intracellular calcium that results from depolarization, but on the mechanism of calcium entry through voltage-sensitive calcium channels. Thus, when calcium entry was stimulated by the calcium ionophore, A23187, P96 and P139 were not dephosphorylated while many of the

increases in phosphorylation for other proteins still occurred. Similarly, a rise in intracellular calcium elicited by the mitochondrial poison guanidine did not result in their dephosphorylation but stimulated other phosphoproteins. Therefore, bypassing the calcium channel prevents P96 and P139 dephosphorylation, but does not prevent the normal increases in labelling of other proteins.

Experiments were then initiated utilizing specific calcium channel agonists and antagonists. These agents selectively affected the phosphorylation of P96 and P139, and only one other protein, P60. Both the agonist BAY K 8644 and the antagonists nifedipine and verapamil elicited large and specific increases in the labelling of these proteins at concentrations below those required to inhibit depolarization-dependent calcium entry. Thus, different procedures aimed at modulating the calcium channel specifically regulate the phosphorylation of these two phosphoproteins. Although the exact identity and function of these proteins remain unknown, the data suggests a role in an aspect of voltage-sensitive ion channel regulation in synaptosomes.

Significance to Biomedical Research and Institute Programs: The results of this research project will improve our understanding of the role of protein phosphorylation in nerve cell function. In particular, the results are an important aid to our understanding of neuronal calcium channels by identifying proteins that may play a role in regulation of the calcium channel. Calcium channel antagonist drugs are becoming important tools in clinical psychiatry yet the molecular mechanisms of their action are not fully understood. This project will provide important information regarding possible molecular mechanisms for the actions of such drugs in nerve cells.

Proposed Course of the Project: The project will be extended in two directions. Firstly, labelled analogues of dihydropyridines will be used to identify the binding proteins in synaptosomes in order to determine whether P96 or P139 might be the binding target of calcium channel drugs. Secondly, the purification of P96 and P139 from brain will be initiated to begin studies on the biochemistry of these two proteins.

Publications

Robinson, P.J., Lovenberg, W., and Dunkley, P.R.: The phosphorylation of two unique synaptosomal proteins, P96 and P139, are differentially regulated by depolarization, cAMP, and calcium channel agonists and antagonists. J. Biol. Chem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03549-01 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Selective Enrichment of Dopamine and Serotonin in Two Populations of Synaptosomes.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Phillip J. Robinson	Visiting Fellow	HE NHLBI
Others:	Donald Kuhn	Staff Fellos	HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new techique has become available for the rapid separation of two distinct populations of synaptosomes from brain. This project examined a variety of parameters to determine whether there might be functional differences between these 2 populations. The content of endogenous dopamine and serotonin differed between the fractions isolated from rat striatum. Fraction 6 contained more serotonin, while fraction 8 contained more dopamine than the corresponding fraction of synaptosomes. Similar extents of release of endogenous neurotransmitters was achieved on depolarization of the two fractions. Maximal rates of activity of the enzyme tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, was not significantly different between the two populations. When the distribution of phosphoproteins in the two populations was examined, the majority of phosphoproteins showed no selective enrichment, while several were selectively localized in one or other population. Two proteins were identified: tyrosine hydroxylase was found selectively enriched in fraction 8, while DARPP-32 was enriched in fraction 6. The data suggests that a relative enrichment of dopaminergic and serotonergic synaptosomes into separate pools has been achieved.

6 of

Objectives: A new technique has been developed for the routine separation of purified synaptosomes into two distinct populations based on differential rates of sedimentation through percoll. However, nothing was known concerning what biochemical or functional differences might exist between the two populations. The aim of this study was to examine the neurotransmitter content of the synaptosomes and determine whether these neurotransmitters could be released on depolarization. The second aim was to determine what phosphoprotein differences might exist between the two populations and to identify certain phosphoproteins that are known to be localized in specific neuronal cell types, in particular, the dopaminergic system.

Methods: Rat striatum was isolated from 6 rats and homogenized in a 10% suspension in 0.32 M sucrose. After centrifugation at 1000 x g for 10 min, the supernatant (S1) was layered onto 8 percoll gradients. The gradients consisted of 4 x 2 ml steps of 23%, 15%, 10%, and 3% percoll (made up in 0.32 M sucrose and 0.25 mM dithiothreitol) and overlayed with 2 ml of S1 (brought to 0.25 mM dithiothreitol), using a peristaltic pump. The gradients were centrifuged at 32,000 x g for 5 minutes at speed and the brake was used to terminate the run. Fraction 6 (at the 10-15% interface) and fraction 8 (15-23% interface) were isolated, diluted at least 3-fold with sucrose and pelleted at 15,000 x g for 30 min. The pellets were washed twice by resuspension in Krebs-bicarbonate buffer pH 7.4 (minus phosphate, 0.1 mM calcium) and centrifugation at 15,000 x g for 10 min, and finally resuspended in 2 ml Krebs and brought to 5 mg/ml protein.

Endogenous dopamine and serotonin content of the synaptosomes was measured by HPLC with electrochemical detection. Tyrosine hydroxylase activity was measured in the presence of maximal biopterin levels using a ^{3}H-tyrosine as substrate. Phospho-tyrosine hydroxylase was determined after prelabelling intact synaptosomes with $^{32}P_i$, by immunoprecipitation of tyrosine hydroxylase with a polyclonal antibody prepared against purified PC12 cell tyrosine hydroxylase. Phospho-DARPP-32 was determined by preparing cytosolic fractions from lysed fraction 6 and 8 synaptosomes and phosphorylation with endogenous ^{32}P-ATP. DARPP-32 was detected by polyacrylamide gel electrophoresis and identified on the criteria that its phosphorylation is uniquely stimulated by cAMP, and that it is exclusively localized in the striatum compared with cortex, cerebellum, or hippocampus.

Major Findings: Striatal fraction 8 synaptosomes contained a significantly greater amount of dopamine (43.6 ng/mg protein) than fraction 6 (29.3), and upon depolarization, endogenous levels were significantly reduced to 84% (fraction 8) and 86% (fraction 6). In contrast, endogenous serotonin levels were greater in fraction 6 (2.46) than fraction 8 (1.39), and upon depolarization levels were reduced to 77 and 72%, respectively. Most of the neurotransmitter released from the synaptosomes was recovered in the extrasynaptosomal buffer as serotonin, or as dopamine plus DOPAC.

Phosphoproteins labelled after prelabelling intact synaptosomes with $^{32}P_i$ were essentially identical in both fractions with a few notable exceptions. At least 5 phosphoproteins showed selective enrichment in fraction 6 and 5 in fraction 8. One of the phosphoproteins enriched in fraction 8 synaptosomes was identified as tyrosine hydroxylase, which demonstrated an approximately 3-fold

enrichment. One of the phosphoproteins enriched in fraction 6 by at least 4-fold was DARPP-32.

Significance to Biomedical Research and Institute Program: The use of percoll gradient centrifugation allowed, for the first time, the routine separation of purified synaptosomes into two distinct populations in a very short time. This project demonstrated that there are several distinct biochemical differences between these two populations. Firstly, there is a major difference in certain neurotransmitter distributions, with the majority of dopamine in fraction 8 and serotonin in fraction 6. The results raise the possibility that a relative enrichment of serotonergic synaptosomes in fraction 6 and of dopaminergic synaptosomes in fraction 8 has been achieved. Secondly, two specific phosphoproteins that have known cellular localizations were demonstrated to be selectively localized in one or other population. Tyrosine hydroxylase is a marker for dopamine synaptosomes in striatum, and its localization in fraction 8 supports the suggestion that dopaminergic synaptosomes are enriched in that fraction. DARPP-32 is a marker for neurons that are post-synaptic to dopamine neurons, and its enrichment in fraction 6 supports the notion that pre- and post-synaptic dopamine terminals may be separated into distinct populations. These results will have important applications to studies of dopamine neurons and their receptors, and may prove to have applications to the study of human diseases such as schizophrenia.

Proposed Course of Project: The project will be extended in several directions. Firstly, the relative enrichment of serotonin in fraction 6 suggests a co-enrichment of tryptophan hydroxylase in that fraction. This enzyme is rate limiting in serotonin synthesis and will be assayed for activity and phosphorylation. The possible separation of pre- and post-synaptic dopamine systems will be pursued by examination of the responses of the two populations to a range of dopamine agonists and antagonist drugs. Finally, a range of other neurotransmitters and known enzymes can be examined for possible enrichment.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03550-01 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Brain Serotonin and Cardiovascular Control

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard McCarty	Senior Fellow	HE NHLBI
Other:	Geoffrey Head	Guest Worker	HE NHLBI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.5	0

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

There is compelling evidence that central serotonin neurons are involved in regulation of the cardiovascular system. However, the brain circuitry which underlies this influence is poorly understood. The major focus of this project is to investigate the effects of discrete neurotoxic lesions of brain serotonin pathways on baroreceptor reflexes in conscious rats. The selective serotonin neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), will be injected into: (a) NTS, (b) medial forebrain bundle, (c) spinal cord or (d) lateral ventricles. Vehicle-injected controls will be included for each group. Within two weeks following microinjections of 5,7-DHT or vehicle, rats will be prepared with chronic arterial and venous catheters to permit direct recordings of blood pressure and heart rate and administration of drugs into conscious, unrestrained rats. Baroreceptor function will be assessed by dose-response curves for sodium nitroprusside (0.5-20 μg/kg) and phenylephrine (0.1-50 μg/kg) in each rat. Our findings to date indicate that baroreceptor function is largely spared following localized or global brain serotonin lesions.

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Objectives: To assess the contribution of brain serotonin neurons to regulation of blood pressure and heart rate, laboratory rats will be studied following selective destruction of central serotonin pathways.

Methods: Stereotaxic microinjections of 5,7-dihydroxytryptamine (5,7-DHT), a serotonin neurotoxin, will be made two weeks prior to testing. Baroreceptor function will be evaluated in conscious rats prepared with chronic arterial and venous catheters. All surgery will be performed in laboratory rats anesthetized with brevital.

Major findings: The use of nitroprusside and phenylephrine provides a convenient and reproducible means for assessing baroreceptor funtion in conscious, unrestrained rats. Baroreceptor curves are similar in animals tested 1 and 2 days after implanting of catheters. In control rats, reflex increases in heart rate induced by i.v. nitroprusside result from increases in sympathetic drive to the heart and from withdrawal of parasympathetic tone to the heart. With reflex decreases in heart rate induced by i.v. phenylephrine, there is almost exclusively an increase in parasympathetic tone to the heart. Surprisingly, brain serotonin lesions had no significant effects on baroreceptor function.

Significance to Biomedical Research and Institute Programs: Defects in baroreceptor function may underlie the onset of essential hypertension in humans. Serotonin, a neurotransmitter, is localized in brain areas known to be involved in baroreceptor function and has effects on blood pressure in laboratory animals. Continued research in this area may contribute to a better understanding of the neuronal pathways involved in maintaining normal cardiovascular homeostasis.

Proposed Course of Project: Termination

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03551-01 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Autoradiographic Analysis of Somatostatin Binding Sites in Brain

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Richard McCarty	Senior Fellow	HE NHLBI
Others:	Laura M. Plunkett	PRAT Fellow	LCS NIMH
	Kazuto Shigematsu	Visiting Scientist	LCS NIMH
	Juan M. Saavedra	Staff Scientist	LCS NIMH

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The biologically active peptide, somatostatin, has been localized in several hypothalamic and extrahypothalamic brain regions where it may function as a classical neurotransmitter or as a modulator of neural activity. We have studied somatostatin binding sites in rat brain by incubation of tissue sections with 125-I-tyr-1-somatostatin, Ultrofilm autoradiography, computerized microdensitometry and comparison with 125-I standards. Highest concentrations of somatostatin binding sites (fmol/mg protein) were found in the claustrum, central nucleus of the amygdala, deep layers of cerebral cortex, lateral olfactory nuclei, hippocampus, medial and lateral septal nuclei, and the medial habenula. Regulation of somatostatin binding sites may be studied as one approach to examining the involvement of brain somatostatin pathways in various physiological and behavioral states.

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Objectives: We sought to examine the localization of binding sites for somatostatin in rat brain as one approach to examining the role of somatostatin in neuronal function.

Methods: Coronal sections of rat brain were incubated with ^{125}I-tyr^{1}-somatostatin and then opposed to Ultrofilm. The films were developed and optical densities of specific areas were measured by a computerized microdensitometer. ^{125}I standards were included with each film and allowed for quantitation of somatostatin binding in discrete brain areas.

Major findings: Somatostatin binding sites were highly localized in specific forebrain areas, but were absent in midbrain and brainstem areas. Highest concentrations of binding sites (fmol/mg protein) were found in the claustrum (151), central nucleus of the amygdala (90), deep layers of the cerebral cortex (61), lateral olfactory nuclei (58), CA1 and CA2 areas of the hippocampus (57), medial and lateral septal nuclei (54), and the medial habenula (44). The distribution of receptors is in general agreement with studies on the distribution of somatostatin immunoreactivity in rat brain. Limbic areas appear to receive projections from hypothalamic neurons. In contrast, receptors within the deep layers of the cerebral cortex and central nucleus of the amygdala appear to be components of intrinsic neuronal systems within these areas.

Significance to Biomedical Research and Institute Programs: Our interests in brain somatostatin binding sites were stimulated by two lines of research. The first concerns several reports of decreased somatostatin immunoreactivity in cerebral cortex in post mortem tissue from patients with Alzheimer's Disease. The second involves a possible role for limbic system somatostatin in cardiovascular responses to stress. Our planned experiments address each of these potential roles for brain somatostatin pathways.

Proposed Course of Project: Termination

Publications:

McCarty, R., Plunkett, L.M., Shigematsu, K., and Saavedra, J.M.: Quantitative autoradiographic analysis of somatostatin binding sites in discrete areas of rat brain. Peptides, 1985, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03552-01 HE

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*
Regulatory Mechanisms for Voltage-dependent Ca^{2+} Channels in Rat Brain.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Enrico Sanna	Guest Scientist	HE NHLBI
Others:	Gabriele Panza	Guest Scientist	HE NHLBI
	Jack Grebb	Clinical Associate	PP NIMH
	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
	Arthur G. Wright	Chemist	HE NHLBI

COOPERATING UNITS *(if any)*
None

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SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

3H-nitrendipine binding sites appear to be a molecular component of voltage-dependent Ca2+ channels located in nerve cell bodies of caudate nuclei. In kainic acid-lesioned caudate nucleus 3H-nitrendipine binding sites and veratridine-elicited increase of 45Ca2+-uptake were abloted. The number of 3H-nitrendipine binding sites was decreased by 40% in caudate nucleus, hippocampus and cortex of mice after nifedipine or verapamil-feeding for 28 days.

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Objectives: In cardiac and smooth muscle cells inward movement of calcium through voltage-dependent channels is thought to initiate the action potential and trigger contraction. Various groups of organic Ca^{2+} channel antagonists have been shown to block the increase in Ca^{2+} current. Recent antiradiographic studies indicated that radiolabelled Ca^{2+} channel antagonists have high affinity binding sites in brain, there they are regionally distributed. A large density ^{3}H-nitrendipine recognition sites was found in olfactory bulb, frontal-panetal cortex, caudate nucleus, and hippocampus, while negligent specific binding occurs in brainstem and cerebellum. Although the existence of recognition sites for Ca^{2+} channel antagonist has been documented by various laboratories, no information is available on their physiological role or their regulatory function for voltage-dependent Ca^{2+} channels in the brain.

This project is designed to obtain information on regulatory mechanism for voltage-deptendent Ca^{2+} channels. Studies will be carried out to determine whether 1) ^{3}H-nitrendipine-recognition sites can be used as biochemical markers for Ca^{2+} channels, and 2) whether Ca^{2+} channels are regulated by a specific endogenous ligand or whether they are associated with multiple neurotransmitter systems?

Methods:

^{3}H-nitrendipine binding is measured in membranes prepared from crude or purified synaptosomes. Non-specific binding is measured in presence of 20 µM nifedipine. The data from saturable binding isotherms are analysed according to Scatchard to obtain the values for K_D and B_{max}.

Voltage-dependent $^{45}Ca^{2+}$ uptake into brain slices or purified synaptosomes preparations is measured in absence and presence of various concentrations of veratridine (range 10^{-6} M to 3 10^{-5} M). In some experiments the addition of tetradotoxin (1/5 [veratridine]) is used to differentiate between voltage-dependent or veratridine-sensitive Ca^{2+} channels.

Chemical lesion of corpus striatum: To destroy dopaminergic and serotonergic nerve terminals, 6-OH-dopamine (250 µg/10 µl), 5,7-dihydroxylryptamine (20 µg/10 µl), respectively, are infused into the lateral ventricle. Kainic acid is injected in the head section (2 µg/1 µl) and tail section (1 µg/.5 µl) of caudate nucleus to obtain destruction of nerve cell bodies.

Major findings:

Down-regulation of ^{3}H-nitrendipine recognition sites: Mice received nifedipine, verapamil, or diltiazem mixed in powdered chow for 28 days and were fed only powdered chow in the last 5 days before killing. ^{3}H-nitrendipine binding to membranes prepared from caudate nucleus, hippocampus, and frontal-panetal cortex was significantly decreased (B_{max} values 40% lower than control) in mice fed nifedipine or verapamil. In contrast, in diltiazem-fed mice neither the B_{max} nor K_D values for ^{3}H-nitrendipine binding differed from controls. Studies of $^{45}Ca^{2+}$ uptake in slices prepared from caudate nucleus, hippocampus, and frontal-panetal cortex showed that the veratridine-elicited increase of $^{45}Ca^{2+}$ was reduced in mice fed nifedipine or verapamil, but not diltiazem. These results

bring evidence that ^{3}H-nitrendipine binding sites may play a physiological role in the function of voltage-dependent Ca^{2+} channels in the brain.

Evidence for specific localization of voltage-dependent Ca^{2+} channels in nerve cell bodies of caudate nucleus. Destruction of dopaminergic or serotonergic nerve terminal in caudate nucleus failed to alter the density of ^{3}H-nitrendipine binding sites. In contrast, when nerve cell bodies were abloted by injection of kainic acid intrastriatally, the number of ^{3}H-nitrendipine binding sites was reduced by 90% to 100%. In addition, in slices of kainic acid-lesioned caudate nuclei veratridine failed to increase $^{45}Ca^{2+}$ uptake.

Significance to Biomedical Research: The goal of this research project is to introduce the concept of association of supra molecular unit of Ca^{2+} channel in the regulation of post-synaptic receptor for neurotransmitters. The outcome of these studies will improve the present knowledge on mechanisms related to transmitter- and cotransmitter-elicited depolarization of post-synaptic neuronal membranes.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1984 to September 30, 1985

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that control transport.

Isolated segments of renal tubules.

In order to understand kidneys at a cellular and molecular level the functions of the different types of epithelial cells must be identified. Progress in this direction has relied heavily on the direct study of individual nephron segments. Each nephron segment has a different cell morphology and function. An important method (which originated in this laboratory) for directly studying the nephron segments is to dissect them and perfuse them individually in vitro. The findings, during the past year, using this method are as follows:

Knepper, Tomita, Pisano, and Burg have been studying the effects of mineralocorticoids, arginine vasopressin, and bradykinin on isolated perfused cortical collecting ducts from rats, because these agents are known to have important effects on urinary electrolyte excretion. The purpose of the studies is to identify the sites and mechanisms of action of the agents within the kidney. Chronic deoxycorticosterone treatment of the rats increased sodium absorption and potassium secretion by their collecting ducts in vitro. Addition of arginine vasopressin to the bath in vitro further increased the rates of sodium, chloride, bicarbonate, and fluid absorption and the rate of potassium secretion. In contrast, addition of bradykinin to the bath in vitro inhibited the sodium and chloride absorption, but did not significantly affect potassium or bicarbonate transport, or the transepithelial potential difference. Thus, the investigators have shown that the diuretic action of bradykinin is due, at least in part, to inhibition of a previously unidentified electroneutral NaCl transport process in rat cortical collecting ducts.

Knepper, Garvin, Star, and Burg have been studying ammonia and bicarbonate transport by isolated, perfused tubules from rats and rabbits, because ammonia and bicarbonate are the principal components of net acid in urine and blood, and are therefore important for renal control of acid-base balance. The results with rabbit cortical collecting ducts were: (1) there was more or less bicarbonate secretion into the urine, depending on the systemic acid-base state of the animals from which the collecting ducts were obtained for in vitro perfusion. (2) Bicarbonate secretion occurred by electroneutral exchange for chloride which was reabsorbed from the urine. (3) There was also proton secretion in parallel with the bicarbonate secretion, and the proton secretion generated an acidic pH disequilibrium in the lumen. (4) The luminal pH

disequilibrium caused ammonia secretion by nonionic diffusion. The results in rat cortical collecting ducts were virtually identical to rabbit, except that the presence of ammonium in perfusate and bath converted bicarbonate secretion to absorption in rat, but not rabbit collecting ducts. In proximal straight tubules from rabbits, ammonium in the bath was found to substitute for potassium in supporting fluid and bicarbonate absorption. Both fluid and bicarbonate absorption were already known to require potassium for exchange with sodium on the Na,K-ATPase. Thus, the new studies provide evidence that ammonium, as well as potassium, is able to exchange with sodium on the Na-K-ATPase.

Knepper has been studying water and urea transport in isolated renal epithelia and mathematically modelling their role in urinary concentration. In the experiments he compared the urea transport in various collecting duct segments. There was no active urea transport in any of the segments. The inner medullary collecting duct was heterogeneous with respect to urea permeability. Collecting ducts from the outer third of the inner medulla had a low permeability to urea, similar to the low permeability previously found in medullary rays and outer medulla. In contrast, collecting ducts from the inner two-thirds of the inner medulla had an extremely high permeability to urea. Mathematical modelling showed that this pattern of collecting duct urea permeability maximizes the axial urea gradient in the inner medulla by delaying urea absorption to near the papillary tip where the effective blood flow is lowest. Knepper and Sands found that the papillary surface epithelium had a low permeability to urea and a small surface area relative to inner medullary collecting ducts. They conclude that, in disagreement with current theories, urea transport across the papillary epithelium is unlikely to contribute significantly to urea delivery to the inner medullary interstitium.

Strange and Spring have developed and utilized a computer controlled, video, light microscope technique to measure the size and shape of the cells in isolated perfused rabbit cortical collecting ducts. By following the rate of change of cell volume in the first seconds after a step change in the concentration of the perfusate or bath, they measured the osmotic water permeability of the apical and basolateral membranes of the two cell types (principal and intercalated) in this epithelium. The basolateral water permeability was very high in both cell types, regardless of the conditions studied. The apical water permeability, on the other hand, was low until stimulated by vasopressin. These are the first precise direct measurements of this important parameter in cortical collecting ducts.

Regulation of amphibian epithelial cell volume and solute transport.

Spring and his colleagues have been studying solute and water transport by the epithelia in the Necturus gall bladder and the frog skin. Because epithelia may transport large quantities of salt and water, their cells are subjected to significant osmotic stress. Therefore, the mechanisms by which epithelial cells minimize changes in size and shape while the rates of fluid entry and exit are changing are important. The investigators have developed and used a unique combination of light microscopic, video, computer, and electrophysiologic methods to study cell volume and intracellular ionic content. They found that

the epithelial cells in these tissues were very leaky to water, and therefore the cell volume was directly dependent on cell solute content. They have now analyzed the factors which control solute movements across the cell membranes.

Foskett and Spring have found that Necturus gallbladder cells contain a number of quiescent transport systems which are transiently activated in response to changes in bathing solution osmolality or solute transport rate. Following osmotically induced swelling, the shrinkage of the epithelial cells back to normal size results from transport of KCl out the basal cell surface, followed by water. This shrinkage is triggered by a process dependent on an intact cytoskeleton and calmodulin. On the other hand, the ability of the cells to swell after osmotic shrinkage does not involve the same triggering factors. It results from flux of NaCl into the cells through newly activated transporters in the apical cell membranes.

Ussing, Foskett and Spring have investigated the route of NaCl transport across frog skin, by using the optical techniques mentioned above and by mapping the ionic currents above individual cells. They found that the sodium and the chloride each pass through different cells. The sodium is transported through the principal cells, always present in the frog skin. Chloride, on the other hand, passes through specialized cells rich in mitochondria. The number of mitochondrial rich cells varies with the demand for NaCl transport.

Cell culture of epithelia.

Although the technique of perfusing kidney tubules in vitro has provided an overall description of their transport properties, it has been difficult to extend the studies to subcellular and molecular levels. Chemical and physical methods for studying transport require much larger amounts of homogeneous tissue than are present in single tubules. Handler, Burg and their colleagues have been using cultures of epithelial cells to overcome this difficulty.

Handler and colleagues have been studying epithelia formed by continuous cell lines derived from kidney. In studies of epithelia formed by A6 cells (from kidney of Xenopus laevis) they previously found functions like those of the cortical collecting duct. Sodium transport by the A6 epithelium (and the cortical collecting duct) was inhibited by amiloride and was stimulated by hormones such as adenosine, isoproterenol, and vasopressin, which activate adenylate cyclase, and by adrenal steroid hormones such as aldosterone. Full differentiation of A6 occurred only when cells were grown on a porous support such as a filter bottom cup that allowed access of medium to the basal surface of the cells, but not if the cells were grown on solid supports, such as standard plastic culture dishes. Preston and Handler have found that the differentiation on filters involves simultaneous appearance of transport, transepithelial resistance, adenylate cyclase response to vasopressin, and morphological maturation. The complex differentiation on filters was dramatically accelerated by the addition of dexamethasone to the culture medium. Yanase and Handler found that the sodium transport by A6 epithelia was inhibited by agents that activate protein kinase C. The inhibition occurred at the entry step for sodium into the cells through their apical plasma membrane. The

investigators also found that cyclic AMP and hormones that raise cell cyclic AMP levels stimulated active secretion of chloride from basal to apical surface. Chloride secretion was not evident without stimulation.

Gstraunthaler and Handler found that LLC-PK1 cells (derived from porcine kidney) formed ammonia and that ammonia formation, as in the proximal tubule of mammalian kidney, increased at low pH. Also as in mammalian kidney, glutamine was the major substrate for ammonia production. Proximal tubules are capable of gluconeogenesis, and the rate of gluconeogenesis is increased as ammonia production increases in acidosis. The investigators initially found that the LLC-PK1 epithelia were incapable of gluconeogenesis, lacking the key enzyme fructose 1,6-bisphosphatase. By growing LLC-PK1 cells in medium without glucose, however, they selected a strain of LLC-PK1 cells that is capable of gluconeogenesis. Comparison of this strain to the wild type will enable them to study the relationship between ammonia formation and gluconeogenesis.

Handler, Spiegel, Fishman, and Turner, have continued to study the polarization which results in different compositions of the apical and basal plasma membranes in epithelia. They had demonstrated in earlier studies that lipids that do not flip-flop from one leaflet of the plasma membrane bilayer to the other, did not diffuse in the plane of the plasma membrane from the apical to the basolateral plasma membrane. Those experiments used exogenous fluorescent tagged lipids, however, and endogenous lipids had not been tested. Therefore, the investigators have extended the studies to a natural glycolipid, the ganglioside GM1. Using the specific binding of cholera toxin to quantify the amount of GM1 on the apical or basolateral plasma membrane, they showed that GM1 which does not flip-flop does not move from apical plasma membrane to the basolateral plasma membrane. Turner and Handler have used monoclonal antibodies to trace an unidentified antigenic apical membrane lipid that is not detectable in the basolateral plasma membrane. They found that the antibody-lipid complex was taken up into the cells by endocytosis and returned to the apical plasma membrane. An unexpected finding was that the endocytosed complex also appeared in the basolateral plasma membrane. The finding indicates that the antibody interferes with the normal sorting mechanism for the lipid.

Green, Burg, S. Guggino, W. Guggino, and Triche have studied the transport function of a line of rabbit medullary thick ascending limb cells (GRB-MAL1) which they originated and have now maintained in continuous culture for more than three years. The tissue has characteristic epithelial morphology on electron microscopy. Its origin from medullary thick ascending limb was initially established by selective dissection of a fragment of medullary thick ascending limb as the starting material and confirmed by finding Tamm-Horsfall protein (which is unique to this segment) in the tissue culture. The present studies utilized patch clamps and cellular impalements with microelectrodes to measure cellular voltages and ion currents. Barium and furosemide changed the cellular voltage in a manner consistent with their known actions on the K^+ channels and Na^+,K^+,Cl^- carriers responsible for transepithelial Na^+, K^+, and Cl^- transport in this tissue. The investigators have used patch clamps to measure currents through individual K^+ channels in the apical membranes of the cells and are characterizing the channels. In addition to the

electrophysiological experiments, the investigators are applying the method that was successful for establishing the MAL culture to grow rabbit renal cortical collecting duct cells.

Uchida, Green, Triche, Coon, Mims and Burg have been studying the effect of hypertonicity on a continuous line of cells (GRB-PAP1) that they originated from rabbit renal papilla. The renal papilla normally is markedly hypertonic, and cells generally require special adaptations to survive such hypertonicity. GRB-PAP1 has been in continuous culture in normal isotonic medium (300 mOsm) for 7 years past 55 passages. A strain of these cells has grown continuously in hypertonic medium (600 mOsm, increased by NaCl added to the isotonic medium) for two years. The cells in hypertonic medium express a distinctive morphological phenotype. Normal rat thyroid cells exposed to similarly hypertonic medium died within two weeks. When a number of clones from GRB-PAP1 were placed in hypertonic media, some cells from each clone survived, showing that the process was adaptation, not selection. Cloning efficiency was used as a measure of adaptation. The cells conditioned to hypertonic medium had a relatively low cloning efficiency in isotonic medium, but a relatively high cloning efficiency in hypertonic medium. The investigators consider that the mechanisms involved in adaptation of papillary cells to hypertonicity may be important for the normal function of the renal papilla which is susceptible to otherwise unexplained necrosis in various conditions. The experiments are continuing with examination of the cells for intracellular osmolytes and for effects of hypertonicity with solutes other than NaCl, particularly urea which is also present in high concentrations in the papilla.

Green, Burg, Bassin, and Coon have been attempting to establish continuous tissue culture lines from rabbit proximal renal tubules. The starting material is a suspension of tubules from rabbit renal cortex that is enriched in proximal tubules. No serum is present in the medium, which prevents the growth of fibroblasts. Proximal tubules are selected in glucose-free medium. Proximal tubules can grow with °-ketoglutarate or maltose in place of glucose because they (but not other kidney cells) have enzymes of gluconeogenesis and maltase. The investigators have not succeeded in growing the cells beyond the fifth passage, however, with or without glucose. In order to extend growth they are transfecting with oncogenes, because some oncogenes are believed to "immortalize" cells in culture. Transfection with v-myc by the calcium phosphate method did not result in prolonged survival, however, and variations of the transfection method and other oncogenes are now being tested.

Metabolism associated with solute transport.

A large fraction of the metabolism of renal epithelial cells is utilized to produce energy for transepithelial transport. Balaban and his coworkers have been using non-invasive nuclear magnetic resonance and optical spectroscopy techniques to investigate the general mechanisms that regulate cellular energy metabolism so that it keeps up with cellular work in renal and, also, in cardiac tissue.

Balaban, Kantor, Briggs, Feretti, Lynch, and Saks studied the role of high

energy phosphate compounds in the control of metabolism by using ^{31}P NMR. In a followup of similar studies in kidneys, they measured the concentration and turnover of these compounds in dog hearts in vivo during alterations in cardiac work output. They found that the concentrations of ATP, ADP, creatinine phosphate and inorganic phosphate did not change during the cardiac cycle or when the work of the heart was changed greatly by altering the heart rate. The result is like the previous one in kidneys. The conclusion is that, contrary to prevailing theory, the concentrations of these compounds do not control energy metabolism in vivo over the ranges of work output investigated. The investigators are continuing with direct studies of metabolism of isolated mitochondria and of epithelial cells in culture in order to discover in these well controlled systems what the feedback mechanism is which paces metabolism to keep up with workload.

Balaban, Kurtz, Smith and Lynch have been using microscope optical spectroscopy to measure intracellular pH and redox state. They built a microscope system capable of resolving detailed fluorescence and transmission spectra at multiple defined sites within a cell while viewing the cellular image. Both structural and spectral information are recorded with a sensitive television camera, digitalized, and analyzed by computer. In the initial studies a fluorescent dye (1,4 dihydroxyphallonitrile) was identified that measures pH when it is introduced into cells as an ester. The validity and accuracy of the pH measurements was established by studies in cultured cells and blastocysts. The method is now being used to study acidification by isolated, perfused renal tubules and to investigate the control of pH in cells in culture.

Bagnasco, Balaban, Fales, and Burg have been studying the osmolytes in rabbit and rat renal inner medulla. As urine is concentrated, the blood in the renal papilla becomes hypertonic because the levels of urea and NaCl are high. The urea concentration is equally high in the cells, but the NaCl remains at a normal low cellular level, apparently because a substantially higher NaCl concentrations would interfere with metabolism. Like other animal cells, those in the renal medulla cannot maintain substantial osmotic pressures across their plasma membranes. Therefore, the excess osmolality of the extracellular NaCl must theoretically be balanced by some other intracellular "osmolytes", but the identity of the osmolytes was not previously known. The investigators have now measured those osmolytes by proton NMR, mass spectroscopy, and specific enzyme-based biochemical determinations. The osmolytes were the same in rabbits and rats, namely betaine, phosphorylglycerolcholine, sorbitol, and inositol. The concentrations of these compounds varied directly with the osmolality of the urine and medullary tissue. Further studies are planned to establish the origin of the osmolytes, how their concentration is controlled, and their status in various pathological conditions.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01224-08 KE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Control of sodium and potassium transport by the nephron

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Mark Knepper	Senior Investigator	LKEM, NHLBI
Other:	Kimio Tomita	Visiting Fellow	PCS, LC, NHLBI
	John Pisano	Chief, PCS	PCS, LC, NHLBI
	Maurice Burg	Chief, LKEM	LKEM, NHLBI

COOPERATING UNITS *(if any)*

Physiological Chemistry Section, Laboratory of Chemistry, NHLBI

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The effects of mineralocorticoids, arginine vasopressin, and bradykinin on isolated perfused cortical collecting ducts from rats are being investigated. Chronic deoxycorticosterone treatment of the rats increases sodium absorption and potassium secretion by the collecting ducts in vitro. Addition of arginine vasopressin to the bath in vitro increases the rates of sodium, chloride, bicarbonate, and fluid absorption and the rate of potassium secretion. Addition of bradykinin to the bath in vitro inhibited the sodium and chloride absorption, but did not significantly affect potassium or bicarbonate transport, or the transepithelial potential difference. These results are consistent with the presence of an electroneutral NaCl transport process in rat cortical collecting ducts that is inhibited by bradykinin.

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Objectives

The chief long term goal of this project is to determine the mechanisms of control of sodium and potassium excretion by the kidney. The current emphasis is on identification of the hormonal signals that may affect electrolyte transport in collecting duct segments.

Methods

Cortical collecting ducts are dissected from rat kidneys and perfused in vitro. Sodium, potassium, chloride, bicarbonate and inulin concentrations are measured in the perfusion fluid, the bathing fluid, and in the collected fluid to determine the fluxes of each substance across the tubule epithelium. Potential difference is measured across the epithelium. Vasopressin (AVP) and/or bradykinin (BK) in physiological concentrations are placed in the bath or perfusate after control measurements are made to determine the effects of the agents on the measured variables. Some of the animals are pretreated with deoxycorticosterone (DOC) to assess the effect of long term exposure to increased levels of adrenal corticosteroids with mineralocorticoid activity.

Results

Vasopressin added to the bath markedly increased both the sodium absorption and the potassium secretion, while also increasing the transepithelial potential difference. This contrasts with prior results in the rabbit where vasopressin markedly inhibited the sodium flux and voltage of isolated cortical collecting ducts.

Bradykinin substantially inhibited the sodium flux without a significant effect on potassium flux or potential difference.

Further studies of anion transport in cortical collecting ducts from deoxycorticosterone-treated rats yielded the following results:

Animal Treatment	In vitro Hormone* (in bath)	Flux (peq/min/mm)		Potential Difference, (mv)
		Cl	HCO_3	
DOC	None	7.1**	-7.3**	-5.4**
	AVP	40.1	2.4	-11.6
DOC	AVP	48.3**	12.2	-20.9
	AVP+BK	29.2	14.2	-20.3

*AVP, 10^{-10} M. BK, 10^{-9} M.
**$p < 0.05$ versus control.

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Significance

The cortical collecting duct is an important site of NaCl reabsorption and potassium secretion. It is a major site of control of urinary electrolyte excretion. Evidence from other types of studies suggests that mineralocorticoids, vasopressin and the kallikrein-kinin system play in important role in the regulation of renal electrolyte excretion. These studies indicate that all three exert important effects on electrolyte transport by the cortical collecting duct of the rat.

Proposed course

Further studies are proposed to test the effects of several diuretic agents on the electroneutral NaCl transport in order to identify the mechanism of coupling of the two ions.

Publications

Tomita, K., J.J. Pisano, and M.A. Knepper. Control of Na+ and K+ transport in the cortical collecting duct of rat: Effects of bradykinin, vasopressin, and deoxycorticosterone. J. Clin. Invest. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01237-07 KE

PERIOD COVERED

October 1, 1984 to September, 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Hormonal control of transport in kidney epithelia in culture.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Masahiro Yanase	Visiting Fellow	LKEM, NHLBI
	Agnes S. Preston	Research Chemist	LKEM, NHLBI
Others:	Jacqueline Muller	Senior Investigator	CDB, DBB
	Chester Williams	Biology Lab. Tech.	LKEM, NHLBI
	R. J. Turner	Associate Professor	Univ. of Toronto
	Joseph S. Handler	Chief, MMS	LKEM, NHLBI

COOPERATING UNITS *(if any)*

University of Toronto, Division of Membrane Biology, Toronto, Canada and Center for Drugs and Biologics, Division of Biochemistry and Biophysics

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Membrane Metabolism Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.50	2.50	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The response to hormones is studied in an epithelium as it forms in culture. Epithelia formed by A6 cells do not respond to vasopressin when grown on a petri dish. When grown on a millipore filter bottom cup, the epithelia respond to vasopressin. There is a close correlation between the development of vasopressin sensitive adenylate cyclase, transepithelial resistance, and formation of a morphologically ordered epithelium. Incubation with dexamethasone accelerates differentiation. In differentiated epithelia, the rate of sodium transport is inhibited by activators of protein kinase C. The inhibition is at the apical entry step for sodium.

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Objectives

The purpose of this study is to examine the cellular factors involved in the hormonal regulation of epithelial sodium transport. Current work is focused on the stimulation of sodium transport in epithelia formed in culture by A6 cells, a continuous line derived from the kidney of Xenopus laevis. We have described the transport properties of the epithelium and its responses to hormones in previous reports. We have also identified culture conditions that lead to epithelia that lack responsiveness to vasopressin, as well as conditions that lead to a marked stimulation of adenylate cyclase by vasopressin and stimulation of sodium transport.

The objective is to identify the cellular changes that occur and the factors that influence these changes as the epithelium becomes responsive to vasopressin. This will lead to a greater understanding of differentiation of epithelial function as well as a better understanding of hormonal control of sodium transport.

Methods

See previous annual reports for methods of cell culture, and methods of measuring sodium transport, cAMP, and adenylate cyclase activity.

Major Findings

Last year we discovered that dexamethasone dramatically accelerated the differentiation of A6 epithelia as estimated by functional markers such as transepithelial electrical resistance, sodium transport, and adenylate cyclase response to vasopressin. This year we established that there is concomitant morphological differentiation. These results indicate that dexamethasone has a profound, broad effect on differentiation of A6 epithelia.

In other studies we have shown that agents such as phorbol esters, diacylglycerol, and mezerein, which activate protein kinase C, inhibit amiloride sensitive sodium transport in A6 epithelia. Since the inhibition is reversed by nystatin added to the solution bathing the apical plasma membrane, it is located at the apical plasma membrane step for sodium entry into the cell. The results indicate that activation of protein kinase C is another mechanism by which amiloride sensitive sodium channels can be regulated.

We have also found that in addition to stimulating apical to basal transport of sodium, cAMP and agents that raise cell cAMP levels stimulate the basal to apical transport (secretion) of chloride. Chloride secretion is inhibited by the addition of bumetanide to the solution bathing the basal surface of the epithelium. Chloride secretion is not detectable under basal conditions.

The polarity of epithelial transport depends on differences in composition and function of their apical and basolateral plasma membranes. We have raised monoclonal antibodies against constituents of the apical plasma membrane of A6 epithelia and have used them to study the maintenance of the polar distribution of membrane constituents. One antibody binds to a lipid in the apical membrane.

It does not bind to the basolateral plasma membrane indicating that the lipid is located only in the apical plasma membrane. After binding to the lipid, approximately 50% of the antibody is endocytosed over a period of two hours. The antibody reappears on the apical surface indicating that it and presumably the lipid antigen are recycled to the plasma membrane after endocytosis. We also found the antibody recycled to the basolateral plasma membrane. Since the lipid antigen is normally not found on the basolateral plasma membrane, antibody binding must have interfered with the normal sorting mechanism for endocytosed membrane.

Proposed Course

We are attempting to isolate the apical plasma membrane using lectin affinity chromatography. The isolated membrane will be used to perform vesicle flux and other studies to examine the amiloride inhibitable sodium channel further. Other monoclonal antibodies will be used to explore polarity.

Publications

Lang, M.A., A.S. Preston, J.S. Handler, and J.N. Forrest, Jr.: Adenosine stimulates sodium transport in kidney (A6) epithelia in culture. Am. J. Physiol. (in press).

Lang, M.A., J. Muller, A.S. Preston, and J.S. Handler.: Complete response to vasopressin requires epithelial organization in A6 cells in culture. Am. J. Physiol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL01246-05 KE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Primary and continuous culture of epithelial kidney cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Maurice Burg	Chief	LKEM, NHLBI
Other:	Nordica Green	Chemist	LKEM, NHLBI
	Shunya Uchida	Visiting Fellow	LKEM, NHLBI
	Hayden Coon		NCI, DCDB, LG
	Timothy Triche		NCI, LP
	Sharon Mims		NCI, LP
	Robert Bassin		NCI, DCBD
	Sandra Guggino		NIA, GRC
	William Guggino		University of Maryland, Baltimore, MD
	John Hoyer		Children's Hospital, Philadelphia, PA

COOPERATING UNITS *(if any)*

National Cancer Institute, University of Maryland and Children's Hospital, Philadelphia, PA

LAB/BRANCH

SECTION

Laboratory of Kidney and Electrolyte Metabolism

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Continuous lines of cells have been established in tissue culture from rabbit renal medullary thick ascending limbs and papillary pelvic epithelium and are being used for studies of the their transport function and resistance to hypertonicity. The same methods are being applied to establish continuous lines from other renal epithelia.

630

Methods

The methods used to establish GRB-MAL1 and GRB-PAP1 were described in previous reports. In brief, fragments containing a few cells from single tubule segments or from papillary pelvic epithelium were dissected and placed in culture. Suitable media and substrata were selected empirically. Use of denuded sheep amnion or collagen coated dishes as supporting structures and supplementation of the medium with defined hormones or pituitary extract were particularly helpful. In addition, proximal tubules are being grown from suspensions of rabbit cortical renal tubules, prepared by mechanical disruption of the kidney, followed by collagenase digestion. Proximal tubules are selected from the other tubule segments by controlling the type of nutrient, in accord with the unique metabolic properties of proximal tubules (e.g. gluconeogenesis and maltase activity). In order to extend the growth of the proximal tubules ("immortalize" them) oncogenes are being introduced by the calcium phosphate precipitate method.

Major findings

1) GRB-MAL1 is now in its third year of culture. A cloned strain is used for study between the 30th and 40th passage. It has characteristic epithelial morphology on electron microscopy. That the origin of the tissue was medullary thick ascending limb was initially established by selective dissection of a fragment of medullary thick ascending limb as the starting material and confirmed by finding Tamm-Horsfall protein (which is unique to this segment) in the tissue culture. The present studies (in collaboration with the Guggino's) utilize patch clamps and cellular impalements with microelectrodes to measure cellular voltages and ion currents. Barium and furosemide change the cellular voltage in a manner consistent with their known actions on the K^+ channels and Na^+,K^+,Cl^- carriers responsible for transepithelial Na^+, K^+, and Cl^- transport in this tissue. Patch clamps have measured currents through individual K^+ channels in the apical membranes of the cells and are being used to characterize the channels. Proposed course: The electrophysiology experiments are continuing. In addition we are applying the method that was successful for establishing the MAL cultures to grow rabbit renal cortical thick ascending limb cells.

2) GRB-PAP1 has been in continuous culture in normal isotonic medium (300 mOsm) for 7 years past 55 passages. A strain of these cells (PAP-HT25) has grown continuously in hypertonic medium (600 mOsm, increased by NaCl added to the isotonic medium) since passage 25. The PAP-HT25 cells express a morphological phenotype ("hyper") different from the GRB-PAP1 cells ("iso"). Both types of cells are epithelial. The PAP-HT25 cells in high NaCl medium, however, are much larger, more heterogeneous, and form epithelia that are in part multilayered, whereas the GRB-PAP1 cells in isotonic medium are small, relatively uniform, and monolayered. When GRB-PAP1 cells were placed in hypertonic medium in passages 25 and 52 most of the cells died, leaving a small population of single cells which propagated and maintained the hyper phenotype. PAP-HT25 cells survived well when switched back from hypertonic to isotonic medium and maintained their hyper phenotype for at least three weeks in isotonic medium. Normal rat thyroid cells in continuous culture died within two weeks of addition of similarly hypertonic medium, indicating that the PAP cells have special adaptive mechanisms. When different clones of GRB-PAP1 cells were placed in hypertonic media, some cells from each clone survived, showing that the process is adaptation, not selection. Cloning efficiency was used as a measure of adaptation. PAP-HT25 cells are at a disadvantage in isotonic medium (cloning efficiency = 4%) compared to GRB-PAP1 cells (cloning efficiency = 12%). The situation is reversed in the hypertonic medium, where the cloning efficiency is 1% for PAP-HT25, but only 0.1 to 0.01% for GRB-PAP1 cells. Proposed course: We consider that the adaptive mechanisms of papillary cells to hypertonicity may be important because this part of the kidney is susceptible to otherwise unexplained necrosis in various conditions. The experiments are continuing with examination of the PAP-HT25 cells for intracellular osmolytes. Also, hypertonicity caused by solutes other than NaCl (particularly urea which is also present in high concentrations in the papilla) is being tested.

3) Proximal tubule cells. We have not yet succeeded in establishing continuous lines from proximal tubules. The starting material is a suspension of tubules from rabbit renal cortex that is enriched in proximal tubules. No serum is present in the medium, which prevents the growth of fibroblasts. Proximal tubules are selected in glucose-free medium. Proximal tubules can grow with °-ketoglutarate or maltose in place of glucose because they (but not other kidney cells) have enzymes of gluconeogenesis and maltase. We have not not succeeded in growing the cells beyond the fifth passage, however, with or without glucose. In order to extend growth we are transfecting with oncogenes, because some oncogenes are believed to "immortalize" cells in culture. Transfection with v-myc by calcium phosphate precipitation did not result in prolonged survival, but other transfection methods and oncogenes are being tested.

Publications

Green, N., A. Algren, J. Houer, T. Triche and M. Burg: Differentiated lines of cells from rabbit renal medullary thick ascending limbs grown on amnion. Am. J. Physiol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01247-05 KE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Title: Urea transport and the urinary concentrating mechanism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: Mark A. Knepper Senior Investigator LKEM, NHLBI

Other: Jeff Sands Med. Staff Fellow LKEM, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies of isolated renal epithelia and mathematical modelling studies are being carried out to investigate the role of urea in the concentrating mechanism and to define the mechanism of concentration in the inner medulla. The inner medullary collecting duct was found to be heterogenous with respect to urea permeability. Collecting ducts from the outer third of the inner medulla have a low permeability to urea like those from the medullary rays and outer medulla. In contrast, collecting ducts from the inner two-thirds of the inner medullary collecting duct have an extremely high permeability to urea, 40 x 10-5 cm/s. This pattern of collecting duct urea permeabilities maximizes the axial urea gradient in the inner medulla by delaying urea absorption to near the papillary tip where the effective blood flow is lowest. The papillary surface epithelium was found to have a low permeability to urea (1 x 10-5 cm/s) and a small surface area relative to inner medullary collecting ducts. It is concluded that urea transport across the papillary epithelium is unlikely to contribute significantly to urea delivery to the inner medullary interstitium.

633

Objectives

The chief long term goal is to define the role that urea plays in the renal concentrating mechanism. The current emphasis is on the mechanism of concentration of the inner medullary interstitium which is the major unresolved issue in the concentrating mechanism field.

Methods

Microdissection techniques are used to isolate the individual epithelial components of the kidney. Isolated nephron segments are perfused in vitro. The epithelium covering the renal papilla is dissected free and mounted in an Ussing-type chamber. Ultramicro methods for the measurement of urea, chloride, creatinine, inulin, and osmolality are used in isolated perfused tubule studies to characterize the transport properties of each nephron component of the kidney. Mathematical modelling techniques are used to analyze the data from the point of view of their overall significance to the aggregate function of the kidney.

Major findings

Urea permeability coefficients have been measured in isolated collecting ducts dissected from five sites along the collecting duct system of the rat. The urea permeability was found to be low in the cortical and outer medullary collecting ducts, extremely high in the inner two-thirds of the inner medullary collecting duct and relatively low in the outer third of the inner medullary collecting duct. This pattern maximizes the axial urea gradient in the inner medulla by delaying urea absorption to near the papillary tip where the effective blood flow is lowest.

It has been proposed by a number of investigators that urea accumulation in the inner medullary interstitium depends in part on passive permeation of urea across the papillary surface epithelium. More recently, it has been proposed by Lory, Gilg, and Horster that urea permeation of this papillary epithelium is an essential component of the counter-current multiplication process in the inner medulla. To test the feasibility of this hypothesis, we have measured both permeability of the papillary epithelium to urea and its surface area. In the rat, the total surface area of all papillary collecting ducts is 86 mm^2. The total surface are of the papillary surface epithelium is only 8.7 mm^2. Thus, the surface area of the papillary surface epithelium is relatively small. We measured the urea permeability of the papillary surface epithelium of the rabbit kidney by dissecting it free from the underlying papillary tissue and mounting it in a small Ussing chamber. The urea permeability measured with C^{14} urea was 1×10^{-5} cm/s or about 40-fold lower than the value in the papillary collecting duct.

Significance

Maintenance of body fluid osmolality is highly dependent on control of water excretion by the kidney. Control of water excretion is in turn dependent on the accumulation of solutes such as urea and sodium chloride in the the renal medulla. Understanding how each nephron segment transports water, urea, sodium chloride, and how these transport processes are regulated in each segment will lead to an improved view of the control of urinary excretion of water.

Proposed course

Studies to define rates and mechanisms of urea, sodium chloride and water transport along the collecting duct system will continue. We will continue to characterize the papillary epithelium in terms of vasopressin response, osmotic water transport and volume regulation.

Publications

Knepper, M.A., and J.L. Stephenson. Urinary concentration and dilution. In: Physiology of Membrane Disorders (2nd edition), edited by T.E. Andreoli, J.F. Hoffman, D.D. Fanestil, and S.G. Schultz. New York: Plenum, 1985 (In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01250-05 KE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Title: Acidification and bicarbonate transport by renal tubules

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Mark Knepper	Senior Investigator	LKEM, NHLBI
Other:	Jeff Garvin	Guest Worker	LKEM, NHLBI
	Robert Star	Med. Staff Fellow	LKEM, NHLBI
	Maurice Burg	Chief, LKEM	LKEM, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.75	2.75	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies of ammonia and bicarbonate transport are being conducted in isolated, perfused tubules from rats and rabbits. The results in rabbit cortical collecting duct showed that: (1) bicarbonate secretion occurs by electroneutral exchange for chloride; (2) bicarbonate secretion is controlled in response to the systemic acid-base state of the animals; (3) proton secretion occurs in parallel to bicarbonate secretion generating an acidic pH disequilibrium in the lumen; and (4) the luminal pH disequilibrium is responsible for ammonia secretion by nonionic diffusion. The results in the rat cortical collecting ducts were qualitatively the same except that the presence of 4 mM total ammonia in perfusate and bath converted bicarbonate secretion to absorption. In the rabbit proximal straight tubule, ammonium was found to substitute for potassium in supporting fluid and bicarbonate absorption indicating that ammonium is able to exchange for sodium on the Na-K-ATPase.

636

Objectives

The chief long term goal of this project is to determine the mechanisms of net acid and buffer transport by the kidney and how these transport processes are controlled. The current emphasis is on ammonia and bicarbonate transport in the proximal tubules and collecting ducts.

Methods

Nephron segments are dissected from rat or rabbit kidneys and perfused in vitro. Total carbon dioxide, total ammonia, chloride, raffinose, creatinine, and/or inulin concentrations are measured in the perfusion fluid, the bathing fluid, and in the collected fluid to determine the fluxes of each substance across the tubule epithelium. Potential difference is measured across the epithelium.

Results

1. Bicarbonate transport by the cortical collecting duct of the rabbit. Chronic deoxycorticosterone administration to the rabbits stimulates a high rate of bicarbonate secretion by the cortical collecting duct in vitro. The bicarbonate secretion is eliminated by chloride replacement on both sides of the epithelium, but not by complete sodium replacement. The bicarbonate secretion is enhanced by chloride removal from the bath and decreased by chloride removal from the lumen. In the presence of ouabain, chloride was reabsorbed actively. Removal of bicarbonate from the bath inhibited the chloride absorption and removal of bicarbonate from the lumen increased the chloride absorption. The rate of bicarbonate secretion does not correlate with transepithelial potential difference. Most likely, the bicarbonate secretion involves neutral bicarbonate-chloride exchange. The metabolic energy transducer ("pump") for the process is as yet unidentified.

2. Ammonia and bicarbonate transport by cortical collecting ducts of the rat. Deoxycorticosterone treatment of the rats results in a high rate of bicarbonate secretion in their cortical collecting ducts as occurs in rabbits. This response is prevented by ammonium chloride treatment of the animals, and enhanced by sodium bicarbonate administration to the animals. Addition of 4 mM total ammonia to the bath and perfusate solutions converted the net bicarbonate secretion to absorption. This contrasts with prior results in the rabbit where the presence of ammonia did not inhibit the bicarbonate secretion. Cortical collecting ducts from deoxycorticosterone treated rats secreted total ammonia, a process that was partially inhibited by carbonic anhydrase addition to the lumen, indicating that the ammonia secretion is in part dependent on a luminal disequilibrium pH. The collected total ammonia concentration was consistent with the value predicted by the diffusion trapping model indicating that nonionic diffusion was the most likely mechanism of ammonia secretion.

3. Ammonia transport in proximal straight tubules. To test whether ammonium can replace potassium in supporting active sodium transport on the Na-K-ATPase, proximal straight tubules were perfused in vitro and fluid absorption and bicarbonate transport were measured. Potassium removal from the solutions inhibited both the fluid absorption and bicarbonate absorption. Transport of both was restored to control levels by addition of 5 mM ammonium chloride to the potassium-free solutions.

Significance

Maintenance of body fluid pH is highly dependent on control of net acid excretion by the kidney. Net acid excretion is dependent on the controlled excretion of protons and the major urinary buffers (bicarbonate, ammonia, and phosphate). Understanding how each nephron segment transports protons and the buffers, and how these transport processes are regulated in each segment will lead to an improved view of the control of urinary excretion of net acid.

Proposed course

Further studies are proposed to measured the permeability of each nephron segment to NH^3 and NH^{4+}, and to carbon dioxide. By use of pH sensitive fluorescent dyes, studies are underway to determine which nephron segments have functional carbonic anhydrase with the catalytic site exposed to the lumen. Mathematical modelling studies will be done to aid in designing experiments, in interpreting the data, and in integrating the results into an overall view of acid-base transport in the kidney.

Publications

Good, D.W., Knepper, M.A., and M.B. Burg. Ammonia and bicarbonate transport by thick ascending limbs of rat kidney. Am. J. Physiol. 247:F35-F44, 1984.

Knepper, M.A., D.W. Good, and M.B. Burg. Mechanism of ammonia secretion by cortical collecting ducts of rabbits. Am. J. Physiol. 247:F729-F738, 1984.

Good, D.W., and M.A. Knepper. Ammonia transport in the mammalian kidney. Am. J. Physiol. 248:Fxxx-Fxxx, 1985.

Good, D. W. Sodium-dependent bicarbonate absorption by cortical thick ascending limb of rat kidney. Am. J. Physiol. 248 (in press).

Garcia-Austt, D. W. Good, M.B. Burg, and M.A. Knepper. Deoxycorticosterone-stimulated bicarbonate secretion in rabbit cortical collecting ducts: Effect of luminal chloride and in vivo acid loading. Am. J. Physiol. 248 (In press).

Good, D.W., M. A. Knepper, and M.B. Burg. Ammonia absorption by the thick ascending limb of Henle's loop. Contr. Nephrol., 1985 (in press).

Knepper, M.A., D.W. Good, and M.B. Burg. Mechanism of ammonia secretion by rabbit cortical collecting ducts: Quantitative considerations. Contr. Nephrol., 1985 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01266-03 KE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Control of epithelial cell volume

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Kenneth R. Spring	Res. Physiologist	LKEM, NHLBI
Other:	Kevin Strange	Guest Worker	LKEM, NHLBI
	Hans Ussing	Guest Worker	LKEM, NHLBI

COOPERATING UNITS *(if any)*

Department of Biological Chemistry, University of Copenhagen

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Large quantities of salt and water move across epithelial cells. These cells are able to maintain a constant volume by balancing solute entry and exit. The mechanisms for epithelial cell volume regulation are under investigation in this laboratory. Optical and microelectrode studies have been performed on the gallbladder of Necturus, on the renal cortical collecting tubule of the rabbit, the frog skin, and on the renal papillary epithelium.

640

GPO 914-918

Objectives

Our goal is to understand the control of cellular volume in epithelia. These tissues transport large quantities of solutes and water across the cell membranes. Previous work from this laboratory has shown that the movement of water can be explained by simple osmotic gradients, and that solute movements are due to transport systems in the apical and basolateral cell membranes. These transporters can be transiently activated when the cell volume is disturbed by an osmolality change. In addition the cells exhibit slow regulatory adjustments to alterations in the rate of solute entry or exit. The mechanisms which sense the volume changes are not known although both calcium and hydrogen ions appear to be involved. The activation of the transport systems has been investigated to some extent but considerably more work needs to be done to fully characterize the changes which occur in response to perturbation of cell volume.

Methods

Over the last ten years we have developed a unique combination of light microscopic, video and electrophysiologic methods which have enabled us to study cell volume and intracellular ionic composition. Briefly, the preparation is visualized with a high resolution light microscope, imaged with a video camera and the images processed and stored. Simultaneous electrical measurement may be made on the cells to determine other functional parameters.

Findings

Our recent work has centered on the factors which are involved in the activation of volume regulation by gallbladder epithelium as well as in the responses of mammalian renal tubules to hormone treatment. We have been able to show that the cell's ability to shrink back to normal size after osmotically-induced swelling depends on an intact cytoskeleton and on calmodulin. The ability of the cell to swell after osmotic shrinkage does not involve these factors and depends on the activation of transporters in the apical cell membrane. We have shown that the transport of Cl across frog skin is accomplished by specialized cells rich in mitochondria. Optical methods have enabled the study of the functional characteristics of the renal papillary epithelium, a tissue which has not been amenable to study by other methods. Our work on renal tubule cells has enabled the first direct measurements of the water permeability of the cell membranes of the cortical collecting tubule.

Proposed Course

We will continue to study renal tubule cells, renal papillary epithelium, frog skin and gallbladder by the techniques described. In addition we will apply more advanced optical and video methods to the analysis of fluorescent as well as bright field images of our preparations.

Publications

Larson, M. and K.R. Spring: Volume regulation in Necturus gallbladder: Basolateral KCl exit. J. Membr. Biol. 81:219-232, 1984.

Jensen, P.K., R.S. Fisher and K.R. Spring: Feedback inhibition of NaCl entry in Necturus gallbladder epithelial cells. J. Membr. Biol. 82:95-104, 1984.

Warnock, D.G., R. Greger, P.B. Dunham, M.A. Benjamin, R.A. Frizzell, M. Field, K.R. Spring, H.E. Ives, P.S. Aronson and J. Seifter: Ion transport processes in apical membranes in epithelia. Fed. Proc. 43:2473-2487, 1984.

Fisher, R.S. and K.R. Spring: Intracellular activities during volume regulation by Necturus gallbladder. J. Membr. Biol. 78:187-199, 1984.

Foskett, J.K. and K.R. Spring: Involvement of calcium and cytoskeleton in gallbladder epithelial cell volume regulation. Am. J. Physiol. 248:C27-C36, 1985.

Kachadorian, W.A., S. Sariban-Sohraby and K.R. Spring: Regulation of water permeability in toad urinary bladder at two barriers. Am. J. Physiol. 248:F260-F265, 1985.

Spring, K.R.: The study of epithelial function by quantitative light microscopy. Pflugers Arch (in press).

Spring, K.R.: Salt and water transport by epithelia: electrophysiologic and light microscopic techniques. Proceedings First International Congress on Comparative Physiology and Biochemistry. (in press).

Marsh, D.J. and K.R. Spring: The polarity of volume regulatory increase by Necturus gallbladder epithelium. Am. J. Physiol. (in press).

Spring, K.R.: Determinants of epithelial cell volume. Fed. Proc. (in press).

Hermansson, K. and K.R. Spring: Potassium induced changes in cell volume of gallbladder epithelium. Am. J. Physiol. (submitted).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01276-01 KE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

The water permeability channel regulated by anti-diuretic hormone.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	William Harris, Jr.	Guest Worker	LKEM, NHLBI
Others:	Helen Murphy	Chemist	LKEM, NHLBI
	James Wade	Assoc. Professor	Univ. Maryland
	Joseph S. Handler	Chief, MMS	LKEM, NHLBI

COOPERATING UNITS *(if any)*

Department of Physiology, University of Maryland, School of Medicine

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Membrane Metabolism Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	1.4	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Aggrephores, the vesicles that are thought to contain water permeability channels that are inserted in the apical plasma membrane in response to vasopressin, are studied in toad urinary bladder with the use of macromolecules with fluorescent tags. Vasopressin elicited endocytosis of the macromolecules is evident and is increased by an osmotic gradient.

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Objectives

The water permeability of toad urinary bladder and mammalian collecting duct is very low under basal conditions and is elevated markedly by vasopressin. In response to vasopressin, intracellular vesicles (aggrephores) that contain particle aggregates (identified only by freeze-fracture electron microscopy), fuse with the impermeable apical plasma membrane and the particle aggregates migrate out into the apical plasma membrane of the epithelial cell. The particle aggregates are believed to contain water permeability channels, and their insertion into the apical plasma membrane is believed to result in the dramatic increase in water permeability elicited by vasopressin. The purpose of this study is to identify, isolate, and characterize aggrephores and particle aggregates from toad urinary bladder.

Methods

In order to label aggrephores, a number of fluorescent and/or radioactive probes have been synthesized. The probes include fluorescent (rhodamine, fluorescein) dextran, fluorescent horseradish peroxidase, and a cleavable radioactive photoaffinity reagent.

Major Findings

Fluorescent dextrans present in the solution bathing the apical surface of the bladder during exposure to vasopressin, are endocytosed when vasopressin is withdrawn. Using two different fluorophores, we have shown that the endocytosed material traverses at least two compartments. The first is believed to be aggrephores returning to the cytoplasm, the second is a lysosomal compartment. Using similar methods, lactoperoxidase has been incorporated into aggrephores and used to iodinate the membrane of aggrephores. The identification and fractionation of these iodinated proteins is in process. Apical membrane proteins have been radiolabeled using several different techniques. 125-I-iodosulfanilic acid labels approximately 30 different membrane proteins in control and vasopressin stimulated bladders.

Proposed Course

Labeling techniques will be modified until a difference is found in bladders that have responded to vasopressin. Aggrephores will be labeled after endocytosing the labeling reagents so that the background contributed by apical membrane constituents that are not involved in the water permeability response will be minimal. Finally, attempts will be made to isolate aggrephores from cell homogenates using endocytosed material to identify aggrephores and differential density gradient centrifugation to separate the aggrephores.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01277-01 KE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cloning the gene for the sodium-coupled hexose cotransporter of epithelia.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Arlyne Garcia-Perez	Guest Worker	LKEM, NHLBI
Others:	Michael Gottesman	Section Chief	LMB, NCI
	Joseph S. Handler	Section Chief	LKEM, NHLBI
	Maurice B. Burg	Laboratory Chief	LKEM, NHLBI

COOPERATING UNITS *(if any)*

Laboratory of Molecular Biology, NCI

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Membrane Metabolism Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.35	1.35	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The project is designed to isolate and study the gene for the sodium-coupled hexose cotransporter of epithelia. Whole genomic DNA from LLC-PK1 cells will be used as starting material.

645

Objectives

The sodium-coupled hexose cotransporter of the proximal tubule has been characterized on the basis of in situ studies and studies in membrane vesicles. It has not been isolated and its structure is not known. The aim of this project is to isolate the gene in order to better understand the function and regulation of the cotransporter.

Methods

Chinese hamster ovary cells (CHO cells) are cotransformed with whole genomic DNA from a renal cell line which expresses the Na-hexose cotransporter, and with pSV2neo, an expression shuttle vector with a dominant selectable marker (G-418 resistance). Surviving clones are screened for expression of the sodium-hexose cotransporter. The screening assay is based on the high affinity of the sodium-coupled hexose cotransporter for alpha-methyl glucopyranoside, a non-metabolizable analog of glucose. Positive clones will be grown out and their DNA used for a second round of transformation of CHO cells to further dilute the number of incorporated genes. Second transformants are screened for expression of the cotransporter. A cosmid library is prepared from DNA purified from positive second transformants.

Major Findings

We have developed a highly specific screening assay for expression of the sodium-hexose cotransporter. The assay uses replica plating on stacks of polyester cloth. The cotransporter is detected by the uptake into CHO cells of alpha-methyl glucopyranoside. Uptake is inhibitable by phlorizin but not by phloretin, and is sodium dependent. The assay is positive for LLC-PK1 cells which are known to express the cotransporter, whereas CHO cells, which are known to lack the cotransporter, are negative.

Proposed Course

Transformation of CHO cells has been started. Assays will be performed to identify transformed clones that express the cotransporter. The general protocol outlined in methods will be followed.

Publications

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01278-01 KE

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ammoniagenesis in cultured renal (LLC-PK1) epithelia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	Gerhard Gstraunthaler	Guest Worker	LKEM, NHLBI
Others:	Joseph S. Handler	Chief, MMS	LKEM, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Membrane Metabolism Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Ammoniagenesis was studied in confluent cultures of the LLC-PK1 renal epithelial cell line. It was found that ammonia production was strictly dependent on the extracellular glutamine concentration. LLC-PK1 cells respond to metabolic acidosis. LLC-PK1 cells exhibited an increase in ammonia production and glutamine consumption with decreasing pH of the culture medium. In order to study the relationship between ammoniagenesis and gluconeogenesis attempts have been undertaken to isolate LLC-PK1 cells capable of gluconeogenesis.

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Objective

The central role of the kidney in maintaining the acid-base balance of mammals depends on its ability to excrete ammonia. In response to metabolic acidosis, the kidney increases production of ammonia. It has been shown that the proximal tubule is the major site of basal urinary ammonia formation and that the adaptive response of ammonia metabolism occurs primarily in the proximal tubule. The renal epithelial cell line LLC-PK1, originated from pig kidney, retains in culture several properties characteristic of the proximal tubule in vivo. Therefore the LLC-PK1 cell line has been chosen to study ammoniagenesis in cultured epithelia. Cultured preparations offer the advantage of being able to study the adaptive response to acidosis without the influence of unrecognized regulatory factors that might be acting in vivo.

Methods

LLC-PK1 cells were routinely grown in plastic culture dishes in Dulbecco's modified Eagle's medium (5 mM glucose, 4 mM glutamine), supplemented with 10% fetal bovine serum, at 37 C in a 5% CO2/95% air incubator for 24 h. Ammonia, glutamine, and glutamate were assayed in the culture medium by enzymatic methods. A gluconeogenic LLC-PK1 strain was isolated as follows: cells were adapted to growth at very low glucose levels. After the adaptation period, confluent cultures were switched to glucose-free medium, supplemented with 10 mM pyruvate. Colonies of LLC-PK1 cells were isolated and propagated in glucose-free medium.

Major Findings

A basic characterization of the ammonia and glutamine metabolism in LLC-PK1 cells revealed the following findings: (1) A strict dependence of ammonia production on the extracellular glutamine concentration. (2) Ammonia production rates are unaffected by glutamate; glutamine consumption, however, decreases with increasing extracellular glutamate. (3) Extracellular hydrolysis of glutamine by gamma-glutamyltranspeptidase does not contribute to ammonia formation. (4) In response to metabolic acidosis, ammonia production and glutamine consumption are increased. (5) As shown previously, the lack of gluconeogenesis in LLC-PK1 cells is due to the lack of fructose-1,6-bis-phosphatase. After adaptation of LLC-PK1 cells to growth in the presence of low glucose levels, colonies of cells capable of growing in the absence of glucose have been isolated. The isolated colonies, propagated in glucose-free medium, expressed fructose-1,6 bisphosphatase activity. (6) Experiments using 3-mercaptopicolinic acid, a specific inhibitor of phosphoenolpyruvate carboxykinase, establish that the adapted strain is capable of gluconeogenesis.

Proposed Course

The strain of gluconeogenic LLC-PK1 cells will be cloned. Using the cloned strain, the relationship between gluconeogenesis and ammoniagenesis will be explored.

Publications

None

Annual Report
Laboratory of Molecular Cardiology
National Heart, Lung, and Blood Institute
October 1, 1984 through September 30, 1985

Actin and myosin are the two major contractile proteins present in smooth muscle as well as non-muscle cells. In smooth muscle and vertebrate non-muscle cells, such as fibroblasts and macrophages, the interaction between actin and myosin that results in contractile activity is regulated by the calcium and calmodulin-dependent phosphorylation of myosin. In contrast, in skeletal and in cardiac muscle this interaction is regulated by a series of proteins that bind to the actin filament, namely troponin and tropomyosin. However, as is the case for smooth muscle and non-muscle cells, initiation of the contractile process is mediated by calcium which, in the case of skeletal and cardiac muscle, binds to troponin C, a protein that is analogous in many ways to the ubiquitous calcium-binding protein, calmodulin. The Laboratory of Molecular Cardiology continues to investigate the regulation of contractile proteins in both smooth muscle and non-muscle cells, as well as in cardiac muscle. The purpose of these investigations is to understand how the contractile proteins are regulated and, particularly, how phosphorylation of myosin, as well as the enzyme, myosin light chain kinase, helps to mediate this regulation. The presence of the contractile proteins, actin and myosin, as well as the enzyme, myosin light chain kinase, in all vertebrate non-muscle cells, suggests that these contractile proteins play an important role in cellular processes such as cytokinesis and, in certain cases, cellular secretion. The Laboratory of Molecular Cardiology continues to investigate the function of contractile proteins in non-muscle cells and we are particularly interested in whether these proteins play a role in both normal, as well as malignant cell division. The laboratory also is investigating the manner by which calcium and the regulatory proteins, troponin and tropomyosin, regulate the ATPase activity of cardiac actomyosin.

Smooth Muscle and Human Platelet Myosin Light Chain Kinase (M. Nishikawa, Departed 4/84; S. Kawamoto, Started 4/85): Prior to returning to Japan, Dr. Masakatsu Nishikawa carried out a comparative study of the phosphorylation of the enzyme, myosin light chain kinase, by cyclic AMP-dependent protein kinase and the calcium-activated, phospholipid-dependent kinase, protein kinase C. He found that protein kinase C could incorporate one mole of phosphate into myosin light chain kinase when calmodulin was bound and two moles of phosphate into myosin light chain kinase when calmodulin was not bound. An exhaustive tryptic digestion of denatured phosphorylated myosin light chain kinase showed that two sites were being phosphorylated by protein kinase C. The site phosphorylated when calmodulin was bound was found to be different than the site phosphorylated by cyclic AMP-dependent protein kinase when calmodulin was bound. On the other hand, the additional site that was phosphorylated when calmodulin was not bound was found to be the same as the additional site that was phosphorylated by cyclic AMP-dependent protein kinase when calmodulin was not bound. Similar to the effects of phos-

phorylation by cyclic AMP-dependent protein kinase, phosphorylation by protein kinase C decreased the affinity of the enzyme for calmodulin. However, the decrease in binding found following phosphorylation by protein kinase C was not as great as that found following phosphorylation by cyclic AMP-dependent protein kinase. This difference in the effect of phosphorylation, as well as the difference in the sites that were phosphorylated by the two kinases, was used to study the mechanism by which phosphorylation altered the binding of calmodulin. It was found that, in the case of cyclic AMP-dependent protein kinase, both phosphorylated sites played a role in weakening the binding of calmodulin to myosin light chain kinase.

Since her arrival in April of 1985, Dr. Sachiyo Kawamoto has been studying myosin and myosin light chain kinase isolated from intact aorta, as well as in aortic smooth muscle cells that have been grown in culture. Using antibodies raised to bovine tracheal myosin, as well as antibodies raised to human platelet myosin, she found that although myosin isolated from the intact aorta was recognized preferentially by antibodies to the tracheal myosin, soon after these cells were placed into culture this myosin binds preferentially to antibodies to human platelet myosin. The question to be resolved is whether this change in the apparent antigenecity of the myosin reflects a switching off of the genes regulating the expression of smooth muscle myosin and switching on of the genes regulating the expression of non-muscle myosin. Dr. Kawamoto is also using antibodies to turkey gizzard myosin light chain kinase to study the cross-reactivity of the different peptides generated by both tryptic digestion and cyanogen bromide cleavage of myosin light chain kinase.

Myosin phosphorylation in non-muscle cells (P. de Lanerolle, Departed 10/84; N. Feuerstein, Started 1/85): Antibodies to human platelet myosin have been used to purify myosin from a number of non-muscle cells, including fibroblasts and lymphocytes. The purpose of these studies is to see whether non-muscle cells grown in culture alter their expression of myosin after the cells are transformed from a "benign" to a more "malignant" state. The potential role that phosphorylation of the light chain and heavy chain of myosin may play in this putative alteration of myosin expression is also under investigation.

The Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction (J.R. Sellers): Dr. Sellers is attempting to determine which step in the kinetic cycle of the hydrolysis of ATP by acto-heavy meromyosin is regulated by phosphorylation. Recent results indicate that phosphate release from acto-heavy meromyosin-ADP-Pi is the step which is regulated by phosphorylation. In collaboration with Dr. Michael Sheetz, University of Connecticut Health Center, and Dr. James Spudich, Stanford University, Dr. Sellers has continued to study the ability of myosin-coated beads to move on an actin substratum of the alga, Nitella. Previous studies have shown that beads coated with smooth-muscle myosin are only capable of moving when the myosin is phosphorylated on the 20,000-dalton light chain. Examination of the movement of beads which were mixed with various ratios of fully phosphorylated and unphosphory-

lated myosin at a constant total myosin concentration showed that the velocity of movement increased with increasing phosphorylation. These results suggest that slowly cycling or non-cycling unphosphorylated myosin cross-bridges may retard the movement of the more rapidly cycling phosphorylated cross-bridges. In addition to studying the phosphorylation of smooth muscle myosin, Dr. Sellers continues to study the phosphorylation of well-defined, proteolytic fragments of myosin isolated from non-muscle sources such as human platelets.

Methylation and Phosphorylation as Regulatory Mechanisms (M. Elizabeth Payne, Departed 5/85; M.A. Conti, Started 4/85): Dr. Payne has carried out amino acid sequence studies on the two sites that are phosphorylated on myosin light chain kinase by cyclic AMP-dependent protein kinase. In collaboration with Dr. Marshall Elzinga at the Brookhaven National Laboratory, a 19-residue peptide has been isolated from turkey gizzard myosin light chain kinase and sequenced. This peptide was shown to contain one of the two serine residues phosphorylated by cyclic AMP-dependent protein kinase. In addition, a six-residue peptide thought to originate at the C-terminus of a 26,000-dalton peptide that contains both phosphorylated sites has also been sequenced and tentatively been identified as containing the second phosphorylated site.

Dr. M.A. Conti has been investigating the possible role of phosphorylation in regulating the maintenance methylase responsible for methylating CpG residues in DNA. Using enzymes supplied by Dr. Timothy Bestor of MIT, Dr. Conti has been using cyclic AMP-dependent protein kinase, myosin light chain kinase and protein kinase C to phosphorylate the methylase in an effort to see whether phosphorylation of the methylase has any effect on the regulation of the activity of this enzyme.

Molecular Genetics: Smooth Muscle Proteins (M. Vahey): Using the expression vector lambda gt 11, Dr. Vahey has isolated 26 cDNA clones which are expressing a peptide recognized by the affinity-purified antibody to myosin light chain kinase. These cDNA clones have been isolated from two different libraries, one having been constructed from mRNA isolated from rat uterus and another from rat aorta cells that have been grown in culture. All of the clones appear to be comprised of 700 base pair inserts and, using restriction endonuclease digestion and electrophoresis on agarose gels, they all appear to be identical. With the cooperation of Dr. Michael Cashel, NICHHD, the cDNA is being sequenced and will be used to isolate larger cDNA clones and subsequently to probe a genomic library for myosin light chain kinase. The ultimate purpose of these investigations is to understand the role and regulation of myosin light chain kinase in smooth muscle and non-muscle cells.

The Regulation of Cardiac Muscle Myosin (L.S. Tobacman): The effect of calcium on the interaction of bovine cardiac myosin subfragment-one with actin in the presence of cardiac troponin-tropomyosin is being studied. Cardiac troponin-tropomyosin mediates a marked calcium-dependent regulation of the steady-state actin-activated MgATPase activity of cardiac myosin S-1. In contrast to a marked regulation of

the ATP hydrolysis there is negligible calcium-dependent regulation of cardiac myosin S-1 binding to actin. The results suggest that in the heart inhibition of the myosin cross-bridge binding is not the predominant mechanism producing myosin relaxation in the absence of calcium. Rather, cardiac relaxation must depend upon marked inhibition of other steps in the cross-bridge cycle. Dr. Tobacman is also studying the rate of ATP hydrolysis in a purified system of actin-myosin and troponin-tropomyosin as a function of calcium concentration. Using values for calcium that he has precisely determined, Dr. Tobacman has measured the progressive activation of the actin-activated ATPase activity with increasing calcium in the presence of regulated actin. The rate increases very cooperatively with calcium, exhibiting a Hill coefficient of 2. This cooperativity is likely to reside solely in the interaction between the adjacent troponin-tropomyosin complexes along the myosin filament; binding of calcium to one complex appears to influence other complexes along the actin filament. Presently, studies are under way to determine the mechanism underlying this cooperative activation of the ATPase activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01665-10 MC

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Smooth Muscle and Human Platelet Myosin Light Chain Kinase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Masakatsu Nishikawa, M.D., Ph.D., Visiting Associate, LMC, NHLBI, Departed 4/84
Sachiyo Kawamoto, Ph.D., Visiting Fellow, LMC, NHLBI, Started 4/85
Robert S. Adelstein, M.D., Chief, LMC, NHLBI
William A. Anderson, Jr., Chemist, LMC, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.8	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Protein kinase C, was used to phosphorylate the calmodulin-dependent enzyme, myosin light chain kinase. Phosphorylation of turkey gizzard myosin light chain kinase, which had calmodulin bound to it, by human platelet protein kinase C, resulted in the incorporation of 1 mole of phosphate per mole of myosin light chain kinase. This phosphorylation had no effect on the activity of myosin light chain kinase. An exhaustive tryptic digestion of the monophosphorylated myosin light chain kinase resulted in a single peptide that occupied a unique position following two-dimensional peptide mapping. This peptide did not co-chromatograph with the peptide that was phosphorylated by cyclic AMP-dependent protein kinase when calmodulin was bound to myosin light chain kinase. When calmodulin was not bound to myosin light chain kinase, protein kinase C incorporated two moles of phosphate per mole of myosin light chain kinase. Diphosphorylation of myosin light chain kinase decreased the activity of the enzyme by decreasing its ability to bind calmodulin. Two-dimensional peptide maps revealed that the additional site that was phosphorylated by protein kinase C in the absence of bound calmodulin was the same as the site that was phosphorylated by cyclic AMP-dependent protein kinase in the absence of bound calmodulin.

Myosin and the enzyme, myosin light chain kinase, are being studied in extracts prepared from intact rat aorta as well as rat aortic cells which are being cultured in vitro. Preliminary experiments using nitrocellulose blots transferred from SDS-polyacrylamide gels indicate that antibodies raised to smooth muscle myosin appear to have a greater affinity for myosin extracted from the intact aorta than they do for myosin extracted from cultured smooth muscle cells.

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Project Description:

Objectives: Phosphorylation of myosin light chain kinase by protein kinase C was studied and compared to phosphorylation of this enzyme by cyclic AMP-dependent protein kinase.

Methods Employed: Enzyme purification using molecular sieve, ion exchange and affinity column chromatography; assays for myosin light chain kinase activity and calmodulin binding; two-dimensional peptide mapping; incorporation of radioactive phosphate into purified proteins.

Major Findings: (1) Protein kinase C phosphorylates myosin light chain kinase with the incorporation of one mole of phosphate per mole of myosin light chain kinase when calmodulin is bound. (2) Protein kinase C incorporates two moles of phosphate into myosin light chain kinase when calmodulin is not bound. (3) The sites phosphorylated by protein kinase C were identified following exhaustive tryptic digestion by 2-D peptide mapping. One of the sites phosphorylated was the same as that phosphorylated by cyclic AMP-dependent protein kinase but one of the sites phosphorylated by protein kinase C appears to be unique. (4) Incorporation of two moles of phosphate into myosin light chain kinase resulted in a decrease in the affinity of this enzyme for calmodulin. (5) By carrying out tandem phosphorylations, using both protein kinase C and cyclic AMP-dependent protein kinase, we were able to ascertain that both sites that were phosphorylated by cyclic AMP-dependent protein kinase had an effect on the ability of the enzyme to bind calmodulin, although only one of the sites that was phosphorylated by protein kinase C seemed to affect the binding of calmodulin.

Significance to Biomedical Research: The regulation of myosin light chain kinase may play a significant role in regulating contractile activity in smooth muscle and non-muscle cells.

Proposed Course: A comparative study is being conducted on the enzyme, myosin light chain kinase as well as myosin isolated from intact smooth muscle cells and smooth muscle cells grown in culture.

Publications:

1. Nishikawa, M., de Lanerolle, P., Lincoln, T.M. and Adelstein, R.S.: Phosphorylation of mammalian myosin light chain kinases by the catalytic subunit of cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase. J. Biol. Chem. 259: 8429-8436, 1984.

2. Nishikawa, M., Sellers, J.R., Adelstein, R.S. and Hidaka, H.: Protein kinase C modullates in vitro phosphorylation of the smooth muscle heavy meromyosin by myosin light chain kinase. J. Biol. Chem. 259: 8808-8814, 1984.

3. Movsesian, M.A., Nishikawa, M. and Adelstein, R.S.: Phosphorylation of phospholamban by calcium-activated, phospholipid-dependent protein kinase. J. Biol. Chem. 259: 8029-8032, 1984.

4. Nishikawa, M., Shirakawa, S. and Adelstein, R.S.: Phosphorylation of smooth muscle myosin light chain kinase by protein kinase C: a comparative study of the phosphorylated sites. J. Biol. Chem. 260: 8978-8983, 1985.

5. Adelstein, R.S., de Lanerolle, P., Nishikawa, M. and Sellers, J.R.: The regulation of smooth muscle contraction by phosphorylation. Elsevier Science Publishers. Vascular Neuroeffector Mechanisms. J.A. Bevan et al. (eds.), 1985, pp. 63-67.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01785-06 MC

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Myosin Phosphorylation in Non-muscle Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Primal de Lanerolle, Ph.D., Departed 10/84
Nili Feuerstein, Ph.D., Visiting Associate, LMC, NHLBI, Started 1/85
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Using an antibody raised to human platelet myosin, myosin has been precipitated from a number of different non-muscle cells. We intend to study whether the myosin purified from non-muscle cells grown in culture is different from myosin that is isolated from cultured cells that have been treated with known mitogens. In particular, we wish to know whether more than one type of myosin is being expressed in a non-muscle cell and whether the type of myosin being expressed in the non-muscle cells varies with cell transformation. The potential role that phosphorylation of the light chain and/or the heavy chain of myosin may play during cell transformation is also under investigation.

656

Project Description:

Objectives: We are interested in studying the role of myosin in non-muscle cells. Previous studies have shown that myosin participates in basic cellular functions such as cytokinesis and, under certain circumstances, cell secretion. Since it is already known that there are a number of genes that control myosin expression the question arises as to whether one form of myosin is associated with normally dividing cells and a different form of myosin may be present in transformed cells. In order to determine if this is the case we will make use of an antibody to human platelet myosin to purify myosin from both normal and transformed cells grown in culture and then, using the techniques of 2-D peptide mapping, determine whether or not there is a change in the myosin isozyme following cell transformation.

Methods Employed: Antibody precipitation; 2-D gel electrophoresis; 2-D peptide mapping.

Major Findings: Work on this project has just recently commenced. There does seem to be some evidence, however, that there may exist in certain cell lines a difference in the myosin light chains between normal and transformed cells.

Significance: Should we be able to demonstrate that a particular isozyme of myosin is associated with cell transformation we then would be in a position to determine at what level (e.g. transcription, etc.) the expression of this myosin is regulated.

Proposed Course: To continue to screen non-muscle cells to see whether or not there is an alteration in the expression of myosin with cell transformation and if so, at what level this alteration takes place.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01786-06 MC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

James R. Sellers, Ph.D., Senior Staff Fellow, LMC, NHLBI
E.V. Harvey, Biologist, LMC, NHLBI
W.A. Anderson, Jr., Chemist, LMC, NHLBI

COOPERATING UNITS *(if any)*

Dr. Mary Pato, University of Saskatchewan
Dr. Michael Sheetz, University of Connecticut Health Center
Dr. Lois Greene, National Heart, Lung, and Blood Institute, NIH

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.0	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The mechanism of the phosphorylation-dependent myosin-linked regulation of smooth muscle myosin is being investigated. This involves several approaches: (1) to identify the rate-limiting step in the acto-myosin MgATPase cycle for both phosphorylated and unphosphorylated heavy meromyosin (HMM), the proteolytic subfragment of myosin; (2) to determine which step(s) is affected by phopshorylation and by how much; (3) to use an in vitro motility system to quantitate how the velocity of movement of myosin-coated beads is affected by various factors, such as calcium concentration, magnesium concentration, and extent of phosphorylation. The mechanism of the phosphorylation-dependent regulation of non-muscle or cytoplasmic myosin is also being investigated.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Objectives: The objective of this project is to understand how phosphorylation regulates the actin-activated MgATPase activity of smooth and non-muscle myosin in vitro which should help elucidate the regulation of smooth muscle and non-muscle contractile processes in vivo. To attack this problem we study how phosphorylation affects the interaction of smooth muscle and non-muscle myosin (or their subfragments) with actin. To do this we use various enzymatic and physical techniques.

Major Findings:

(1) Elucidation of the Regulated Step in the Acto-myosin MgATPase Cycle: Phosphorylation of myosin by myosin light chain (MLC) kinase is required for contraction of smooth muscle. We have attempted to determine which step in the kinetic cycle of the hydrolysis of ATP by acto-HMM is regulated by phosphorylation. Previous experiments from this laboratory have indicated that regulation does not occur at the level of the interaction of HMM·ATP (or HMM·ADP·P_i) with actin. Rather, new results indicate that phosphate (P_i) release from acto-HMM·ADP·P_i is the step which is regulated by phosphorylation. This was determined by transient kinetic experiments using turbidity as a probe for P_i release. Turbidity can be used since HMM·ATP and HMM·ADP·P_i bind weakly to actin while HMM·ADP binds strongly. When ATP is added in stoichiometric amounts to unphosphorylated acto-HMM there is a rapid dissociation followed by a very slow reassociation (approximately 0.002 s^{-1}). The rate of this reassociation is not affected by increasing the actin concentration and is approximately equal to the rate of phosphate release measured directly for unphosphorylated HMM in the absence of actin.

(2) Analysis of the Motility of Smooth Muscle Myosin-coated Beads in an In Vitro System: In collaboration with Dr. Michael Sheetz (University of Connecticut Health Center) and Dr. James Spudich (Stanford University) we have continued to study the ability of myosin-coated beads to move on the actin substratum of the alga, Nitella. Previously, we found that the movement of smooth muscle myosin in this system was dependent upon phosphorylation of the 20,000 Da light chain (i.e. fully phosphorylated myosin-coated beads moved at a constant velocity whereas fully unphosphorylated myosin did not move). An analysis of the concentration dependence of movement of phosphorylated myosin-coated beads showed that, above a myosin concentration of about 7.5 ug/ml, the velocity was constant, and below this value the beads did not move. We have now examined the movement of beads which were mixed with various ratios of fully (i.e. di-) phosphorylated and unphosphorylated myosin at a constant total myosin concentration. Under these conditions the velocity of movement increased with increasing phosphorylation. This behavior is in contrast to the behavior described above for the concentration dependency of velocity of fully phosphorylated myosin and suggests that slowly cycling or non-cycling unphosphorylated myosin cross-bridges retards the movement of the more rapid cycling phosphorylated cross-bridges. The unphosphorylated myosin cross-bridges which are able to bind to actin and create a resistance to movement may be analogous to the so-called "latch bridges" which have been reported to exist in some intact smooth muscle preparations.

(3) Effect of Phosphorylation on the Binding of Smooth Muscle Myosin·ADP To Actin (With Lois Greene, LCB, NHLBI): It was shown above that relaxation of smooth muscle appears to be caused by inhibition of a kinetic step in the actomyosin MgATPase cycle, the step associated with P_i release. A similar step is thought to be blocked in skeletal muscle actomyosin in the absence of Ca^{2+} by the troponin-tropomyosin complex. In this later system troponin-tropomyosin also inhibits the binding of S-1·ADP to actin, raising the possibility that the two phenomena are coupled in some way. In the present study we determined whether dephosphorylation of smooth muscle HMM also affects both the P_i release step and the binding of HMM·ADP to actin using competition studies where phosphorylated and unphosphorylated HMM are allowed to compete for actin sites in the presence of ADP. The data show that phosphorylation only has a small effect on the binding, indicating that this step and P_i release are not necessarily coupled. These results suggest that in smooth muscle, the dephosphorylated myosin inhibited at the step associated with the release of P_i both in the forward and reverse direction.

(4) Preparation of Soluble, Enzymatically Active Subfragments of Non-muscle Myosin: While the MgATPase activity of cytoplasmic or non-muscle myosin is regulated by phosphorylation, little is known concerning the mechanism of this regulation. In order to study this in more detail, we have sought to prepare S-1 and HMM from human platelet myosin. Preliminary results indicate that both subfragments can be prepared. S-1 is produced by papain digestion of platelet myosin at high ionic strength. The S-1 heavy chain (97 kDa) undergoes a secondary proteolysis at a site about 70 kDa from the N-terminus. The cleavage rate at this site is markedly reduced in the presence of actin which should allow for the isolation of an S-1 with an "intact" heavy chain.

HMM is prepared by chymotryptic digestion of phosphorylated myosin in the presence of MgATP. Virtually all of the soluble HMM produced has undergone cleavage at the same internal site discussed above, and thus migrates on SDS gels as two bands at M_r approximately 66 kDa and 64 kDa. The HMM has an actin-activatable MgATPase with a V_{max} of 1 s^{-1} and a K_m for actin of 1.5-3 uM. The low K_m is markedly different from the higher values seen with smooth muscle HMM. Like smooth muscle HMM the K_m of platelet HMM is not greatly weakened by increasing the ionic strength.

Proposed Course: We intend to actively study the mechanism of the phosphorylation-dependent regulation of both smooth and non-muscle myosin using these and other approaches. We are particularly interested in whether phosphorylation affects ADP release in smooth muscle myosin and in whether the general kinetic scheme of non-muscle myosin is similar to that from smooth muscle, particularly with regard to regulation. We are interested in purifying and characterizing myosin from avian intestinal epithelial brush border in order to be able to make detailed comparison of the properties of a smooth muscle and cytoplasmic myosin from the same species.

In addition we hope to develop the Sheetz-Spudich *in vitro* motility assay to quantitatively study effects of ionic conditions and phosphorylation on the velocity of myosin movement on actin.

Publications:

1. Sellers, J.R. and Pato, M.D. The binding of smooth muscle myosin light chain kinase and phosphatases to actin and myosin. J. Biol. Chem. 259: 7740-7746, 1984.

2. Sellers, J.R. and Harvey, E.V. Purification of *Limulus* myosin light chain kinase. Biochemistry 23: 5821-5826, 1984.

3. Margossian, S.S., Chantler, P.D., Sellers, J.R., Malhotra, A., Stafford, W.F. and Slayter, M.S. Susceptibility of both isolated and bound light chains from various myosins to myopathic hamster protease. J. Biol. Chem. 259: 13534-13540, 1984.

4. Sellers, J.R. and Harvey, E.V. Localization of a light chain binding site on smooth muscle myosin revealed by light chain overlay of SDS-polyacrylamide electrophoretic gels. J. Biol. Chem. 259: 14203-14207, 1984.

5. Sellers, J.R. and Adelstein, R.S. The mechanism of regulation of smooth muscle myosin by phosphorylation. Current Topics in Cellular Regulation. (Vol. 27, in press.)

6. Sussman, D.J., Sellers, J.R., Flicker, P.F., Lai, E.Y., Cannon, L.E., Szent-Gyorgyi, A.G., and Fulton, C. Actin of *Naegleria gruberi*. J. Biol. Chem. 259: 7349-7354, 1984.

7. Sellers, J.R., Spudich, J.A. and Sheetz, M.D. Light chain phosphorylation regulates the movement of smooth muscle myosin on actin filaments. J. Cell Biol. (In press.)

8. Daniel, J.L. and Sellers, J.R. Purification and characterization of platelet myosin. Methods in Enzymology. (In press.)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04202-04 MC

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Phosphorylation as a Regulatory Mechanism

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

M. Elizabeth Payne, Ph.D., Staff Fellow, LMC, NHLBI, Departed 5/31/85
Mary Anne Conti, Ph.D., Research Chemist, LMC, NHLBI, Started
Robert S. Adelstein, M.D., Chief, LMC, NHLBI
William A. Anderson, Jr., Chemist, LMC, NHLBI

COOPERATING UNITS *(if any)*

Dr. Marshall Elzinga, Brookhaven National Laboratory
Dr. Timothy Bestor, Massachusetts Institute of Technology

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	1.3	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A 26,000-dalton peptide that contains both sites phosphorylated by cyclic AMP-dependent protein kinase has been isolated from turkey gizzard myosin light chain kinase. Following cleavage with S. aureus protease, it was shown that the first 19 residues from the amino-terminus of this peptide contained one of the two phosphorylated sites and had the following sequence:

-lysine-alanine-phosphoserine-glycine-serine-serine-proline-threonine-serine-proline-isoleucine-asparagine-alanine-aspartic-acid-lysine-valine-glutamic acid-asparagine-glutamic acid-

In addition, a second peptide, which presumably was isolated from the C-terminal portion of the 26,000-dalton paptide contained the following tentative sequence:

-leucine-proline-phosphoserine-proline-valine-lysine-isoleucine-

Presently, studies are under way to determine which of these two peptides is phosphorylated when calmodulin is bound to myosin light chain kinase and which is phosphorylated only when calmodulin is not bound to myosin light chain kinase.

An enzyme that methylates DNA CpG sequences has been purified by Dr. Timothy Bestor (MIT, Cambridge, Massachusetts) and is being used as a potential substrate for a number of kinases. Myosin light chain kinase, protein kinase C and cyclic AMP-dependent protein kinase are each being used in an effort to phosphorylate the DNA methylase.

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Project Description:

Objectives: The primary objective of this study was to sequence the sites that are phosphorylated by cyclic AMP-dependent protein kinase in the enzyme, myosin light chain kinase. This goal has been essentially accomplished and presently we are determining which of the sites is phosphorylated when calmodulin is bound and which site is the unique site that is phosphorylated when calmodulin is not bound. We are also attempting to determine the location of each of the phosphorylated sites in a 26,000-dalton peptide, that has been isolated from myosin light chain kinase and appears to contain both phosphorylated sites.

An enzyme that catalyzes methylation of the DNA sequence CpG has been purified by Dr. Timothy Bestor and is presently being used as a substrate for a number of different kinases. If it is found that the methylase can serve as a substrate for one of these kinases we will study whether or not phosphorylation occurs at a unique site on the methylase and whether this phosphorylation alters the activity of the methylase.

Methods Employed: Enzyme purification using molecular sieve, ion exchange, and affinity column chromatography; high performance liquid chromatography; peptide purification; amino acid sequencing and amino acid analysis; incorporation of radioactive phosphate into purified proteins.

Major Findings: One of the sites phosphorylated by cyclic AMP-dependent protein kinase in the enzyme, myosin light chain kinase, has been determined. It had the following amino acid sequence:

-lysine-alanine-phosphoserine-glycine-serine-serine-proline-
-threonine-serine-proline-isoleucine-asparagine-alanine-aspartic
-acid-lysine-valine-glutamic acid-asparagine-glutamic acid-.

This sequence is known to begin at the amino-terminus of a 26,000-dalton protein that is readily isolated from myosin light chain kinase following brief tryptic digestion of the native enzyme.

A second tentative sequence has been determined for the other site phosphorylated by cyclic AMP-dependent protein kinase. This sequence is:

-leucine-proline-phosphoserine-proline-valine-lysine-isoleucine-.

This sequence is thought to be present in the C-terminal portion of the 26,000-dalton peptide.

Significance to Biomedical Research: These investigations are directed toward obtaining a greater understanding of the relationship between structure and function of myosin light chain kinase. Myosin light chain kinase is an important enzyme in smooth muscle in that it catalyzes the phosphorylation of the 20,000-dalton subunit of myosin. This phosphorylation is thought to be essential for the initiation of smooth muscle contraction.

An understanding of the mechanisms involved in regulating myosin light chain kinase activity is thus essential for understanding how smooth muscle functions.

DNA methylation is thought to play a role in gene expression. The manner in which this enzyme is regulated is, therefore, of potential importance in our understanding of the regulation of gene expression.

Proposed Course: Studies are under way to identify which site on myosin light chain kinase is phosphorylated when calmodulin is bound, and which is the additional site that is phosphorylated when calmodulin is not bound to myosin light chain kinase. The sequence is phosphorylated and will then be compared to other known sites phosphorylated by cyclic AMP-dependent protein kinase.

Studies are in progress to determine if a number of kinases are capable of phosphorylating the DNA methylase supplied to us by Dr. Timothy Bestor. If a unique site on this enzyme is found to undergo a significant amount of phosphorylation, we will study the effects of this phosphorylation on the activity of the enzyme. An effort will then be made to see whether or not this phosphorylation takes place on under in vivo conditions.

Publications:

1. Movsesian, M.A., Swain, A.L. and Adelstein, R.S.: Inhibition of turkey gizzard myosin light chain kinase activity by dihydropyridine calcium agonists. Biochem. Pharmacol. 33: 3759-3764, 1984.

2. Movsesian, M.A. and Adelstein, R.S.: Inhibition of turkey gizzard myosin light chain kinase activity by BAY K 8644. Eur. J. Pharmacol. 103: 161-163, 1984.

3. Movsesian, M.A., Ambudkar, I.S., Adelstein, R.S. and Shamoo, A.E.: Stimulation of canine cardiac sarcoplasmic reticulum Ca^{2+} uptake by dihydropyridine Ca^{2+} antagonists. Biochem. Pharmacol. 34: 195-201, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04205-03 MC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Genetics: Smooth Muscle Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

Maryanne Vahey, Ph.D., Staff Fellow, LMC, NHLBI
Yvette Preston, Biologist, LMC, NHLBI
Robert S. Adelstein, M.D., Chief, LMC, NHLBI

COOPERATING UNITS *(if any)*

Dr. Michael Cashel, Laboratory of Genetics, NICHHD

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Twenty-six presumptive positive copy DNAs, (cDNA) from two smooth muscle lambda gt 11 expression libraries, have been isolated using affinity purified antibody to myosin light chain kinase. The clones appear to be identical with respect to molecular weight of the insert, restriction maps of the insert as well as with respect to preliminary nucleotide sequence of insert ends.

The cDNA has been subcloned into a plasmid vector to facilitate characterization of its messenger RNA (mRNA) as well as further screening of cDNA libraries, genomic libraries and comparison with known amino acid sequence of myosin light chain kinase protein.

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Project Description:

Objectives: The goal of this phase of the project is to purify and characterize cDNA clones presumed to be complementary to the protein myosin light chain kinase. This characterization includes: (1) determination of insert size; (2) relatedness of insert; (3) partial restriction maps of insert; (4) nucleotide sequence of insert; (5) northern blot analysis; and (6) dot blot analysis.

Methods: The rat uterus cDNA library was constructed in lambda gt 11 using reverse transcriptase, S-1 digestion, addition of Eco R-1 polylinkers and infection of Y1090 cells. The rat aorta library is from a cell line and is donated by Dr. Mark Taubman (Harvard Medical School).

The recombinant fusion protein is detected by reaction with affinity purified antibody to gizzard myosin light chain kinase using biotin-avidin horseraddish peroxidase second antibody.

Restriction endonuclease digestion and electrophoresis on agarose gels was used to determine insert size and patterns of various restriction sites within the insert. For the latter study, insert DNA was end-labeled with radionucleotide.

Southern and Northern analysis was carried out under stringent conditions with purified random primer radiolabeled insert.

The Sanger-dideoxynucleotide system was used to determine sequence from both ends of the insert DNA and from exonuclease III generated fragments of interior insert DNA.

The insert was subcloned into the high copy number plasmid pHC 624 (obtained from Dr. Michael Cashel, NICHHD) for further analysis and cDNA library screening.

Results: Antibody screening yielded nine positive clones from the rat uterus library and 17 positive clones from the aorta library. Digestion with Eco R-1 indicated all clones contained a 700 base pair (b.p.) insert. Southern analysis and partial restriction maps indicated that the inserts are identical. While nucleotide sequence analysis of the insert is continuing, approximately 450-500 b.p. determined so far are identical regardless of library of origin.

Dot blot analysis with insert DNA and RNA from several sources suggests: (1) strong hybridization with cardiac, uterine and aorta cell RNA; and (2) less strong hybridization with gizzard, trachea and skeletal muscle RNA.

Preliminary northern analysis suggests the insert DNA hybridizes with a moderate to high molecular weight RNA.

Objectives: The finding that all cDNA clones selected by the antibody screening are identical, suggests there exists a strong relationship between a highly conserved and very specific epitope of the myosin light chain kinase protein and the 700 b.p. insert. Therefore, we have subcloned the 700 b.p. insert into a plasmid vector in order to: (1) rescreen our cDNA libraries and obtain larger, unique and hopefully overlapping clones; (2) probe genomic libraries for the myosin light chain kinase gene; and (3) study the relationship of insert DNA to known amino acid sequence of myosin light chain kinase.

Publications:

1. Schneider, M.D., Sellers, J.R., Vahey, M., Preston, Y.A. and Adelstein, R.S. Localization and topography of antigenic domains within the heavy chain of smooth muscle myosin. J. Cell Biology 101: 66-72, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04206-03 MC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Regulation of Cardiac Myosin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Larry S. Tobacman, M.D., Medical Staff Fellow, LMC, NHLBI
Robert S. Adelstein, M.D., Chief, LMC, NHLBI
William A. Anderson, Jr., Chemist, LMC, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have determined that Ca^{2+} does not alter the affinity of bovine cardiac myosin subfragment-1 (S-1) for actin regulated by cardiac troponin-tropomyosin. Under conditions where the addition of Ca-2+ mediated a 27-fold stimulation of the actin-activated MgATPase rate, Ca-2+ effected negligible change in the binding between the two proteins producing this ATP hydrolysis, actin and myosin S-1. This suggests that the predominant mechanism producing cardiac contraction is not Ca-2+ facilitated binding of myosin cross-bridges to the actin filament. Rather, regulation of cardiac contraction primarily depends upon control of other parts of the cross-bridge cycle.

We have also investigated the MgATPase of cardiac myosin S-1 and regulated actin at intermediate Ca-2+ concentrations. The MgATPase rate increases very cooperatively with increasing Ca-2+ concentration. This cooperativity appears to reside solely in interactions between adjacent troponin-tropomyosin complexes along the actin filament.

66P

Project Description:

Objectives: This study seeks to elucidate molecular mechanisms regulating the interaction of the cardiac contractile proteins, actin, myosin, troponin, and tropomyosin. These proteins are directly responsible for muscle contraction and its regulation. Modulation of their interactions relates directly to modulation of cardiac contractility.

Major Findings: We have evaluated the effect of Ca^{2+} on the interaction of bovine cardiac myosin subfragment-1 (S-1) with actin regulated by cardiac troponin-tropomyosin. Cardiac troponin-tropomyosin mediated a profound Ca^{2+}-dependent regulation of the steady-state actin-activated MgATPase rate of myosin S-1. The extrapolated values for maximal ATP turnover rate at saturating actin, V_{max}, were 6.5 s^{-1} in the presence of Ca^{2+} and 0.24 s^{-1} in the absence of Ca^{2+}. In contrast to this 27-fold regulation of ATP hydrolysis, there was negligible Ca^{2+}-dependent regulation of cardiac myosin S-1 binding to actin. In the presence of ATP, the dissociation constant of regulated actin and cardiac myosin S-1 was 32 uM in the presence of Ca^{2+} and 40 uM in the presence of EGTA. These results suggest that in the heart, inhibition of myosin cross-bridge binding is not the predominant mechanism producing muscle relaxation in the absence of Ca^{2+}. Rather, cardiac relaxation must critically depend upon marked inhibition of other parts of the cross-bridge cycle, evidenced here by almost complete inhibition of the myosin S-1 MgATPase rate when the myosin S-1 is actin-bound.

Since the forcefulness of cardiac contraction is determined and limited by the intracellular Ca^{2+} concentration, we are also studying the rate of ATP hydrolysis in our purified system as a function of Ca^{2+} concentration. This requires precise experimental control of the Ca^{2+} concentration under the conditions needed to measure the ATPase rate. To achieve this control we used EGTA as a Ca^{2+} buffer and measured by a novel method the needed association constants of EGTA for Ca^{2+} and Mg^{2+}. This method utilized the Ca^{2+}-sensitive fluorescent molecule Quin2. By a series of titrations and competition studies using Quin2, Ca^{2+}, Sr^{2+}, Mg^{2+} and EGTA, the desired association constants were determined. Using these values, it was possible to measure the progressive activation with increasing Ca^{2+}, of the myosin S-1 ATPase rate in the presence of regulated actin. The rate increased very cooperatively with Ca^{2+}, exhibiting a Hill coefficient of 2. It is significant that this cooperativity, previously reported with skeletal myosin and regulated actin, also occurred in our system in which two possible sources of cooperativity were removed. Cardiac troponin contains only one regulatory Ca^{2+} binding site, whereas skeletal troponin contains two. Therefore the cooperativity we observed was not due to interactions between such sites. Furthermore, the cooperative activation was not due to cooperative binding of myosin which was excluded by our experimental design. Rather the cooperativity is likely to reside solely in interactions between adjacent troponin-tropomyosin complexes along the actin filament; binding of Ca^{2+} to one complex appears to influence other complexes along the actin filament.

Proposed Course:

(1) Functional comparisons will be made between cardiac troponin-T isoforms. It has been suggested that troponin-T mediates the cooperative activation by Ca^{2+} of the regulated actin filament.

(2) The effect of Ca^{2+} on the binding of heavy meromyosin to the thin filament will be evaluated.

(3) A direct comparison will be made between Ca^{2+}-induced thin filament activation with cardiac troponin C (one regulatory Ca^{2+} site) and activtion with skeletal troponin C (two regulatory Ca^{2+} sites).

(4) The effect of myosin light chain phosphorylation on activation of the cardiac thin filament will be investigated.

(5) An evaluation of the effect of two new inotropic agents on Ca^{2+}-induced activation of the cardiac thin filament will be conducted.

Publications:

1. Tobacman, L.S. and Adelstein, R.S. Enzymatic comparisons between light chain isozymes of human cardiac myosin subfragment-1. J. Biol. Chem. 259: 11226-11230, 1984.

Molecular Disease Branch
National Heart, Lung, and Blood Institute
October 1, 1984 through September 30, 1985

The overall objective of the research program of the Molecular Disease Branch is the delineation of the molecular and structural properties of the human plasma apolipoproteins, the physiological role of the apolipoproteins and lipoproteins in lipid transport, the determination of the mechanisms involved in the regulation of cellular cholesterol metabolism and transport, and the elucidation of the metabolic and molecular mechanisms involved in plasma lipoprotein synthesis, transport, and catabolism in normal individuals and patients with disorders of lipid metabolism and atherosclerosis.

During the last several years the staff of the Molecular Disease Branch has developed a conceptual framework for the understanding of the dynamic processes involved in the biosynthesis, transport, and catabolism of plasma apolipoproteins and lipoproteins. Within this framework the plasma lipoproteins are conceptualized as a polydisperse collection of lipoproteins, the apolipoprotein composition of which is determined by the laws of mass action. The constituent of the lipoprotein particle which is responsible for the regulation of the lipoprotein particle transport and metabolism is the apolipoprotein moeity. The distribution of a specific apolipoprotein within plasma is governed by the relative concentration of and affinity for the individual plasma lipoproteins. This concept of plasma lipoproteins emphasizes the fundamental importance of the apolipoprotein in regulating metabolism and provides a framework for understanding apolipoprotein-lipoprotein interactions during lipoprotein biosynthesis, transport, and catabolism in normal man and in patients with dyslipoproteinemia and atherosclerosis.

The elucidation of specific physiological and biochemical functions of the apolipoproteins continues to be of pivotal importance in our ultimate understanding of the role of apolipoproteins in lipoprotein structure, function, and metabolism. Based on our current information, apolipoproteins have been shown to be of importance in four general facets of lipoprotein metabolism: 1) cofactor for enzymes (apoC-II and apoH for lipoprotein lipase, apoA-I for lecithin cholesterol acyltransferase); 2) ligand on the lipoprotein particle for interaction with high affinity receptor sites (apoB-100 on LDL and apoE on the chylomicron remnant); 3) exchange protein for phospholipids, cholesteryl esters, and triglycerides; and 4) structural component for the lipoprotein particle (apoA-I for HDL, apoB-100 for LDL, and apoB-48 for the chylomicron remnant).

Prerequisite to our understanding of the physiological and biochemical role of apolipoproteins in lipid and lipoprotein metabolism is a detailed knowledge of the molecular structure and function of the plasma apolipoproteins. Over the last several years we have systematically evaluated the molecular structure and primary amino acid sequence of apolipoprotein (apo) A-I, A-II, apoC-I, apoC-II, apoC-III, and apoH.

Of particular interest during the last year is the detailed analysis of the isoproteins of apoB. The two apoB isoproteins, designated apoB-100 and apoB-48, are synthesized by the liver and intestine, respectively. We have recently definitively established that only apoB-100 is synthesized by the human hepatocytes in vitro. Normal human hepatocytes were incubated with radiolabeled amino acids, and the secreted apolipoproteins analyzed by ultracentrifugation, immunoprecipitation with monoclonal antibodies, and NaDodSO$_4$ gel electrophoresis. The only apoB isoprotein secreted by the human hepatocyte was apoB-100. These studies indicate that the apoB-100 and apoB-48 isoproteins can be used as apolipoprotein markers during kinetic studies of lipoprotein metabolism for lipoproteins secreted by the liver and intestine, respectively.

Of major importance during the last year has been the cloning of human liver apoB-100 cDNA in a λgt-11 expression library. The apoB-100 mRNA is 15-18 kb which is of sufficient size to code for an apolipoprotein of 250,000 to 387,000 daltons, the proposed molecular weight of delipidated plasma apoB-100. The cloning of apoB-100 has established that apoB-100 is a very large protein, and has therefore ended the long controversy whether apoB-100 is a very large protein or a small protein covalently linked into a large oligomeric unit.

The complete nucleotide and derived amino acid sequence of a 1.6 kb cDNA clone was determined. The computer predicted secondary structure of the apoB-100 peptide revealed that the majority of the apolipoprotein was organized into beta structure. The large percentage of beta structure may be important in the lipid-apoB-100 interactions on LDL and may contribute to the insolubility of delipidated apoB-100 in aqueous solution.

The gene for apoB-100 was localized to chromosome 2 by hybridization of a cDNA probe for apoB-100 and a panel of mouse-human hybrids. This completes the genomic organization of the two ligands, apoB-100 and apoE, for LDL and the LDL receptor. ApoB-100 localization to chromosome 2 is in contrast to apoE and the LDL receptor which are both present on chromosome 19. Thus apoB-100 is not in synteny with the LDL receptor. Initial studies on the apoB-100 gene in abetalipoproteinemia have been completed and analyses of leukocyte DNA by Southern blot revealed that the apoB-100 gene is present. Northern blot analysis indicated that the mRNA is present and similar in size to the mRNA of apoB-100 in normal subjects. Immunological analyses of plasma and liver biopsy specimens utilizing a monoclonal apoB-100 antiserum have revealed detectable apoB-100 protein. Thus, the defect in abetalipoproteinemia is due to an abnormality in translation or post-translational processing of apoB-100. This is in contrast to the previously held concept that the defect in abetalipoproteinemia was due to a defect in transcription of the apoB-100 gene.

The cloning, structural analysis, and chromosomal localization of apoB-100 have provided new insights into this unique apolipoprotein, and will permit detailed structural analysis of the gene(s) for apoB-48 as well as apoB-100, and the analysis of the apoB-100 gene in patients with dyslipoproteinemias.

During the last year, studies have continued on the gene for apoC-II. ApoC-II is the cofactor for lipoprotein lipase, the enzyme which catalyzes the hydrolysis of plasma triglycerides. The complete amino acid sequence of apoC-II isolated from very low density lipoproteins (VLDL) was reported last year. The complete cDNA sequence and derived amino acid sequence of apoC-II has been completed. ApoC-II is synthesized as a 110 amino acid preapolipoprotein which undergoes co-translational cleavage to the 79 amino acid mature apoC-II which is secreted into plasma.

ApoC-II is located primarily on VLDL and high density lipoproteins (HDL). To gain further insight into the structure - function requirements for apoC-II activation of lipase, synthetic fragments of residue 7-79 and 52-79 were synthesized by the solid phase method. The complete apolipoprotein (residues 1 to 79) and the 7-79 fragment had complete biological activity, however, the 52-79 fragment had little activity indicating that the structural region of apoC-II involved in lipase activation resides in the amino terminal end of the apolipoprotein.

Patients have been identified with a deficiency of apoC-II which clinically is manifested as severe hypertriglyceridemia and a type I hyperlipoproteinemia. In these patients apoC-II has not been detected in plasma and injection of apoC-II into apoC-II deficient patients results in a prompt reduction in plasma triglycerides. The apoC-II gene has recently been evaluated in two kindreds of these patients by restriction enzyme analysis. The apoC-II gene was present, and there are no major insertions or deletions in the apoC-II gene.

Detailed studies on the plasma apolipoproteins have been performed in one of the kindreds with apoC-II deficiency. In this kindred an apoC-II variant, designated apoC-II$_{Bethesda}$, has been identified which has an apparently lower molecular weight by NaDodSO$_4$ gel electrophoresis and a more acid pI. The father and mother of the proband have one half normal plasma levels of apoC-II, however, the mother had the apoC-II$_{Bethesda}$ variant in addition to normal apoC-II. These results were interpreted as indicating that the mother has a normal and C-II$_{Bethesda}$ allele, while the father has a normal and null allele for apoC-II. The proband is the first described case of a compound heterozygote for a defect in the apoC-II gene. Additional studies are currently underway to determine the specific molecular defect in the apoC-II gene in this kindred with apoC-II deficiency.

A detailed analysis of the apoA-II gene has been completed. The cDNA of apoA-II was initially cloned, and the complete cDNA sequence of apoA-II determined. Utilizing apoA-II cDNA probes, the apoA-II gene was identified in a genomic library and the nucleotide sequence of the complete apoA-II gene determined. The apoA-II gene, which resides on chromosome 1, contains three introns of 182, 293, and 395 nucleotides. The first intron is in the 5' untranslated region, the second separates the pre and propeptides, and the third intron is in the middle of the mature apolipoprotein. CAT and TATA boxes were identified in the 5' region of the gene. The genomic organization of apoA-II with three introns is similar to the organization

of the genes for apoC-III and apoA-I suggesting that these three apolipoproteins originate from an ancestral gene.

ApoE as outlined above has been proposed to play a major role in the catabolism of triglyceride rich lipoproteins by interacting with a high affinity receptor in the liver. A deficiency of apoE is associated with hyperlipidemia, a type III lipoproteinemia, and premature cardiovascular disease. The defect in the proband with apoE deficiency has been evaluated by the analysis of the apoE gene and mRNA in plasma leukocytes and macrophages, respectively. ApoE cDNA probes were obtained from apoE cDNA clones previously isolated from a normal liver cDNA library. Southern blot analysis of leukocyte DNA from the apoE deficient patient revealed no major insertions or deletions in the apoE gene. Northern blot analysis of macrophage mRNA detected lesser than 1% of normal apoE mRNA which was similar in size to normal apoE mRNA. Thus, the defect in apoE deficiency is due to a defect in transcription of the apoE gene or the synthesis of an unstable mRNA. Further studies on the structure of the apoE gene isolated from a genomic DNA library are underway to determine the defect in the apoE gene.

During the last year, major advances have been made in our understanding of the biosynthesis and processing of apoA-I, the major protein of HDL. An understanding of the factors which modulate the plasma levels and functions of apoA-I and apoA-II and therefore HDL is of major importance because of the established inverse relationship between premature coronary artery disease and plasma HDL levels.

Previously we have established that apoA-I is synthesized as a 267 amino acid precursor protein, preproapoA-I. Eighteen amino acids are present in the prepeptide and 6 amino acids in the propeptide. During biosynthesis preproapoA-I undergoes co-translational cleavage to proapoA-I. ProapoA-I is secreted from the cells and undergoes post-translational extracellular cleavage to mature apoA-I.

Recently studies have also established a second post-translational modification of apoA-I. The biosynthesis of apoA-I has been studied in human liver HepG-2 cells. Incubation of HepG-2 cells with ^{14}C-palmitate and analysis of the newly secreted apoA-I revealed that apoA-I was acylated. The discovery that human apolipoproteins are acylated is of particular importance since acylation may play a significant role in apolipoprotein-lipid interactions, and the metabolism of apolipoproteins as well as lipoproteins.

During the last few years a significant focus of the Branch has been an analysis of the interaction of plasma lipoproteins and cellular receptors. Previous studies have shown that apoB is important as the major ligand for the interaction of LDL with receptors on peripheral cells, and apoE modulates the uptake of chylomicron remnants by the liver. Recent studies in the Branch have focused on the importance of the liver receptors in lipoprotein metabolism both in normal subjects and patients with dyslipoproteinemias and atherosclerosis. Techniques were developed for culturing of normal human hepatocytes obtained during surgery. In

addition, human hepatic membranes were prepared for analysis of binding without cellular uptake.

A detailed analysis of LDL binding and degradation was performed on liver and fibroblasts grown in vitro. In addition, binding studies were also carried out in liver cells obtained from patients with familial hypercholesterolemia (FH) which have a defective or absence of the LDL receptor. LDL binding was reduced to 50% in hepatocytes from FH patients, however, LDL binding was totally absent in fibroblasts isolated from FH patients. The normal hepatic LDL binding also differed from LDL binding to fibroblast in being calcium resistant, insensitive to protease digestion, and reduced Kd. These combined results establish that there is a different LDL receptor system in hepatocytes than in peripheral cells. The persistence of 50% hepatic binding of LDL in FH patients indicates that there is a hepatic LDL receptor independent of the high affinity LDL receptor. This additional receptor system may be of major importance as a pathway for the slow clearance of plasma LDL in the FH homozygote.

The type of hepatic receptors for plasma lipoproteins was also studied employing lipoproteins containing apoB, apoE, or apoA-I in hepatic membranes from liver tissues isolated from normal controls, FH homozygotes, and a patient with abetalipoproteinemia. ApoB-100 LDL binding was absent in FH, and increased in abetalipoproteinemia when compared to normal LDL binding. Following porta-caval shunt, apoE and apoA-I binding increased and decreased, respectively. These results combined with previous results have been interpreted as indicating that the liver has separate independent receptors for apoB, apoE, and apoA-I containing lipoproteins. The receptors appear to be under separate physiological and genetic control.

The intracellular transport, hydrolysis, and biosynthesis of cholesterol continues to be an active area of research within the Branch. The interaction and uptake of LDL by the LDL receptor initiates a series of biochemical processes leading to the hydrolysis of the cholesteryl esters by acid ester hydrolase with the production of free cholesterol. Free cholesterol down regulates 3-hydroxy-3-methylglutaryl coenzyme reductase (HMG-CoA reductase), the rate-limiting enzyme in cholesterol biosynthesis and activates acyl-cholesterol acyltransferase, the enzyme which reesterifies cholesterol to cholesterol ester. Neutral ester hydrolase is the cytosolic enzyme which is responsible for the hydrolysis of cholesteryl esters formed by the action of acyl-cholesterol acyltransferase. New sensitive enzymic techniques have been developed in the Branch for the quantitation of the enzymic activity of acid ester hydrolase and neutral ester hydrolase. These methods were used in the analysis of these enzymes in fibroblasts grown in culture from patients with Wolman's disease and cholesteryl ester storage disease (CESD). Wolman's disease is characterized by steatorrhea, hepatosplenomegaly, absence of acid ester hydrolase, and death in early infancy. CESD is a milder disease with hepatosplenomegaly and reduced levels of acid ester hydrolase. Previous studies detailed last year have established that the acid ester hydrolase activity was virtually absent in extracts from fibroblasts from both Wolman patients and CESD. Neutral cholesteryl ester activity was present, not reduced, indicating that the two enzymes are independently synthesized.

Additional studies revealed that in CESD the neutral cholesteryl ester activity was increased and may contribute to the milder clinical course observed in CESD patients.

A second major intracellular enzyme involved in cholesterol metabolism is HMG-CoA reductase. The regulation of the enzymic activity of HMG-CoA reductase has been extensively studied in our laboratory over the last several years. HMG-CoA reductase has been purified to homogeneity from chicken, rat, and human liver. In all species now studied, HMG-CoA reductase was shown to be present in enzymically active and inactive forms. The reversible inactivation of HMG-CoA reductase was shown to be due to covalent modification of the enzyme by a reversible phosphorylation-dephosphorylation reaction sequence. The enzyme reductase kinase, which catalyzes the phosphorylation of HMG-CoA reductase, has also been purified to homogeneity. Reductase kinase, like HMG-CoA reductase, was shown to undergo reversible activation-inactivation due to reversible phosphorylation. The kinase responsible for the reversible phosphorylation of reductase kinase has been designated reductase kinase kinase. Regulation of the enzymic activity of HMG-CoA reductase by a bicyclic cascade system provides a rapid short-term mechanism for the regulation of cholesterol biosynthesis.

Modulation of the degree of phosphorylation and enzymic activity of HMG-CoA reductase has been of particular interest, and last year we reported initial studies of a new kinase, protein kinase C. Protein kinase C, which requires calcium and phospholipids, was shown to modulate the enzymic activity of HMG-CoA reductase. Protein kinase C catalyzed the reversible phosphorylation of HMG-CoA reductase, which was associated with a reduction in enzymic activity. In addition, the tumor-promoting phorbol ester, phorbol 12 myristate 13 acetate (PMA), stimulated the protein kinase C catalyzed phosphorylation of HMG-CoA reductase. These results suggest a possible in vivo protein kinase C-mediated mechanism for the short-term regulation of the enzymic activity of HMG-CoA reductase.

A second new kinase which is a calcium, calmodulin dependent protein kinase (CMK) has been identified which reversibly modulates the enzymic activity of HMG-CoA reductase. CMK, purified from rat brain cytosol, reversible phosphorylated both native microsomal and purified soluble HMG-CoA reductase. The phosphorylation of HMG-CoA reductase was associated with a reduction in enzymic activity; dephosphorylation with a phosphoprotein phosphatase restored HMG-CoA reductase enzymic activity.

The identification of the CMK system in addition to the reductase kinase and C kinase systems for the modulation of the enzymic activity of HMG-CoA reductase provides new insights into the molecular mechanisms involved in the regulation of cholesterol biosynthesis.

The synthesis, transport, and catabolism of plasma lipoproteins in normal subjects and patients with dyslipoproteinemia continues to be an active area of investigation within the Branch. An important area of research in the Branch continues to be focused on the metabolism of HDL, since HDL has been clearly identified as a negative risk factor for the

development of cardiovascular disease. Previous studies have been performed on HDL, apoA-I, and apoA-II. Of major interest are our recent studies which have established that proapoA-I is secreted into plasma and lymph and undergoes extracellular post-translational cleavage to mature apoA-I in plasma. Recent data have established that the conversion of proapoA-I to mature apoA-I is quantitative, and there is no direct catabolism of proapoA-I. In plasma from fasting subjects, approximately 5% of total apoA-I is proapoA-I. Studies have also continued on the analysis of apoA-I metabolism in Tangier disease. Tangier disease is characterized by orange tonsils, lymphadenopathy, hepatosplenomegaly, recurrent peripheral neuropathy, and severe deficiency of plasma HDL, apoA-I, and apoA-II. The gene for apoA-I was cloned from the liver of a patient with Tangier disease. The complete cDNA sequence of the apoA-I gene from a Tangier patient was determined, and was identical to the gene of normal apoA-I except for the substitution of a aspartic acid for a glutamic acid at position 120 in the sequence.

Studies were also performed on plasma apoA-I from patients with Tangier disease. Two forms of apoA-I were identified, and purified. One form was identical to normal apoA-I on $NaDodSO_4$ gel electrophoresis and isoelectrofocusing whereas the other form was 2,000 daltons smaller in molecular weight and had a more basic pI. Kinetic analysis of the two forms of apoA-I Tangier in normal controls revealed the first form to be catabolized at the same rate as normal apoA-I, whereas the smaller molecular weight form of apoA-I Tangier was rapidly catabolized. These combined results have been interpreted as indicating that the defect in Tangier disease is a post-translational processing abnormality which results in the formation of an apoA-I which is rapidly catabolized leading to a marked deficiency of apoA-I and HDL.

One of the informative areas of research over the last several years has been the analysis of the metabolism of apoE. ApoE is coded for by three major alleles, E^2, E^3, and E^4, and several lines of *in vitro* and *in vivo* metabolic evidence suggest that the normal allele is E^3. Previous studies from our Branch have established that the product of the E^2 allele, $apoE_2$, is catabolized more slowly than $apoE_3$. These results are consistent with the delayed catabolism of remnants of triglyceride-rich lipoproteins characteristic of patients with type III hyperlipoproteinemia. The metabolism of $apoE_2$ was also extended to normolipidemic subjects homozygous for $apoE_2$. Initial studies established that normolipidemic $apoE_2$ homozygotes have a two- to threefold elevation of plasma apoE and an increase in cholesterol-rich VLDL. Analysis of apoE metabolism in these subjects revealed that the increase in plasma apoE was due to increase in synthesis.

Recent studies on apoE metabolism have focused on the molecular mechanisms involved in the rapid catabolism of $apoE_4$, and the delayed catabolism of $apoE_2$ when compared to the catabolism of $apoE_3$. $ApoE_2$ was modified by the addition of methyl and aminoethyl groups to the free sulfhydryl groups to block the reactive sulfhydryl group and to convert the sulfhydryl groups to neutral and positive charges, respectively. Aminoethyl-$apoE_2$ was catabolized at the same rate as $apoE_4$, while

methyl-apoE$_2$ was catabolized at a rate intermediate between apoE$_2$ and apoE$_4$. These results are consistent with the principle difference in catabolism of apoE$_2$ and apoE$_4$ being the two positive charges in apoE$_4$. Modification of apoE$_2$ to add two positive charges via aminoethylation resulted in the formation of an apoE$_2$ derivative which was catabolized at the same rate as apoE$_4$. The elimination of the two reactive sulfhydryl groups may also have altered apoE$_2$ catabolism due to inability of the modified apoE$_2$ to form mixed disulfide oligomers.

The role of the carbohydrate moeity in the catabolism of apoE is as yet unknown. The predominate isoform of apoE$_3$ in plasma contains no carbohydrate, however, mono and disialo forms of apoE are present in plasma. Asialo and disialo forms of apoE were isolated by preparative isoelectrofocussing, and the kinetics of the two apoE forms studied in vivo in normal control subjects. Both forms of apoE were catabolized at a similar rate, and there was no conversion of the disailo to the asialo form of apoE. These results indicate that either there is no conversion of the carbohydrate to noncarbohydrate containing apoE, or that the conversion taken place on a newly secreted particle or cellular space which is not accessible to the apoE injected into the plasma. Further studies will be required to establish the relative importance and the site of removal of the carbohydrate moeity of apoE.

One of the major aims of the staff of the Branch is the effective treatment of hyperlipidemia with the ultimate goal of reducing blood lipid levels at an early stage of atherosclerosis and preventing premature cardiovascular disease. To this end we have initiated an ongoing outpatient clinical trial for the treatment of patients with hypercholesterolemia and the type II phenotype with neomycin and neomycin-nicotinic combination. Neomycin reduced total and LDL cholesterol levels an average of 20 to 24%, respectively. No ototoxicity, nephrotoxicity, or serious side effects have been detected over a two-year period. Neomycin in combination with nicotinic acid normalized the plasma lipoprotein levels in 92% of the patients with type II hyperlipoproteinemia.

Recently we have initiated a double blind clinical trail on mevinolin, a drug which inhibits HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis. Initial results of a drug regimen of 25 mg bid indicate that the majority of type II patients may normalize plasma LDL with mevinolin therapy. This medication has the potential to be the most effective single drug therapy for patients with type II hyperlipoproteinemia available today.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02010-14 MDB

PERIOD COVERED: October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Structure and Function of Plasma Lipoproteins and Apolipoproteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	H. Bryan Brewer, Jr., M.D.	Chief	MDB. NHLBI
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	A. Hospattankar, Ph.D.	Visiting Associate	MDB, NHLBI
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COOPERATING UNITS *(if any)*:

LAB/BRANCH: Molecular Disease Branch

SECTION: Peptide Chemistry

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.3	2.3	3.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human plasma apolipoprotein (apo) A-I has been shown to be synthesized as a preproapolipoprotein. ProapoA-I undergoes post-translational proteolytic processing to mature apoA-I. In addition, apoA-I has been shown to undergo post-translational acylation with fatty acids. The biosynthesis and processing of apoA-I involves several different types of processing including proteolysis as well as modification with different prosthetic groups. The processing of apolipoproteins may play an important role in the metabolism as well as physiological function of the plasma apolipoproteins and lipoproteins.

The complete covalent structure of apoC-III has been determined in normal subjects. This structure differed from that previously determined from a patient with type V hyperlipoproteinemia. These results suggests that structural variants of apolipoproteins may be present which are important in the pathophysiology of the human dyslipoproteinemias.

Two different isoforms of apoA-I have been isolated from Tangier patients. One form of apoA-I was electrophoretically as well as kinetically similar to normal apoA-I. The other isoform had an apparent molecular weight which was 2000 daltons less than normal apoA-I, and was rapidly catabolized when injected into normal controls. These results indicate that the defect in Tangier disease is due to an abnormality in post-translational processing of apoA-I leading to reduced levels of HDL.

ApoB-100 has been cloned in an λgt-11 expression vector. The expressed fusion protein contains a 60,000 dalton apoB-100 protein which reacted with a monoclonal antibody that is specific for the LDL receptor binding site. The cloned apoB-100 contains the LDL receptor binding domain, and will permit detailed studies on the interaction of apoB-100 and the LDL receptor.

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Project Description

Objective:

1) Isolation, characterization, and amino acid sequence of human apoC-III.

Methods Employed:

ApoC-III was isolated from delipidated plasma lipoproteins (d <1.006 g/ml) obtained from normal subjects and patients with type V hyperlipoproteinemia. ApoC-III was purified to homogeneity by gel permeation chromatography on Sephacyl S-200 followed by ion exchange chromatography on DE-52. Tryptic peptides of apoC-III were isolated by ion exchange chromatography on DE-52 utilizing volatile buffers. The isolated peptides and apolipoproteins were analyzed for purity by thin layer chromatography, $NaDodSO_4$ gel electrophoresis, and amino-terminal analysis.

Major Findings

ApoC-III was a single electrophoretic band on $NaDodSO_4$ gel electrophoresis, and had a single amino-terminal amino acid serine. The complete sequence of apoC-III was determined by automated Edman aminoterminal analysis of the intact apolipoprotein, and isolated tryptic peptide. Automated Edman degradation of the intact apolipoprotein were continued for 41 cycles, thus establishing the amino acid sequence of the first half of the apolipoprotein. The amino acid sequence was completed by sequence analysis of the 7 tryptic peptides isolated from apoC-III. The complete amino acid sequence of apoC-III from normal subjects differed from our previous structural analysis of apoC-III isolated from a patient with type V hyperlipoproteinemia at 4 positions including residues 32 (glu for ser), 33 (ser for gln), 37 (gln for ala) and 39 (ala for gln). The complete amino acid sequence of apoC-III from a second type V patient was identical to normal apoC-III. These results are interpreted as indicating that structural variants of apoC-III are present in at least some patients with type V hyperlipoproteinemia, and these variants may be of importance in the pathogenesis of the dyslipoproteinemia.

Objective:

2) Analysis of post-translational modification of human apoA-I.

Methods Employed:

Human apoA-I was analyzed following secretion from the human hepatoma cell line HEP-G2. HEP-G2 cells were incubated with uniformly labeled ^{14}C-palmitate and 1-^{14}C-palmitate to evaluate post-translational modification of apoA-I by acylation. The acylated apoA-I was evaluated following $NaDodSO_4$ gel as well as 2 dimensional gel electrophoresis, by hydroxylamine treatment, and immunoprecipitation with a monospecific apoA-I antiserum. The potential

for removal of acyl group on apoA-I by serum, and heparinized as well as EDTA plasma was investigated by incubation at 37°C for 24 hrs followed by $NaDodSO_4$ gel electrophoresis.

Major Findings:

Characterization of nascent apoA-I secreted into the media of HEP-G2 cells incubated with ^{14}C-palmitate revealed that apoA-I was acylated with palmitate when analyzed by $NaDodSO_4$ gel electrophoresis and autoradiography. The acylation of apoA-I with palmitate was confirmed by immunoprecipitation with an apoA-I antiserum, and hydroxylamine treatment result in the virtual complete loss of the ^{14}C-palmitate from apoA-I indicating that the acyl group(s) was covalently linked to apoA-I. Several of the apoA-I isoforms were shown to be acylated when nascent apoA-I was analyzed by 2 dimensional gel electrophoresis. ^{14}C-oleate was not incorporated into secreted nascent apoA-I establishing the specificity of the fatty acid acylation of apoA-I. Incubation of ^{14}C-palmitate acylated apoA-I in serum or plasma did not result in deacylation. These results establish that apolipoproteins undergo acylation, and suggest that the covalent linkage of lipids to apolipoproteins may play a critical role in apolipoprotein and lipoprotein metabolism.

Objective:

3) Characterization of apoA-I isoforms in Tangier patients.

Methods Employed:

ApoA-I isoforms from the plasma of patients with Tangier disease were isolated by affinity chromatography with a monospecific apoA-I antiserum, or by chromatography on phenylsepharose followed by preparative isoelectrofocussing. Normal apoA-I isoforms were prepared from delipidated high density lipoproteins by preparative isoelectrofocussing. Isolated isoforms were analyzed for purity by analytical isoelectrofocusing, and $NaDodSO_4$ gel electrophoresis.

Major Findings:

ApoA-I present in Tangier plasma can be separated by $NaDodSO_4$ gel electrophoresis into two forms which differ in apparent molecular weight by approximately 2,000. The two isoforms of $apoA\text{-}I_{Tangier}$ also differ by an approximately 1 charge shift on electrofocussing. The two isoforms were purified to homogeneity as outlined above, and were a single electrophoretic band on $NaDodSO_4$ gel electrophoresis as well as a single component on 2-dimensional gel electrophoresis. The kinetics of radiolabeled $apoA\text{-}I_{Tangier}$ isoforms were studied in normal volunteers over 7 days by the techniques outlined in previous annual reports. The $apoA\text{-}I_{Tangier}$ isoform of apparently normal molecular weight was catabolized at a normal rate, whereas the $apoA\text{-}I_{Tangier}$ isoform of lower apparent molecular weight was rapidly catabolized. These results are consistent

with the defect in Tangier disease being due to a post-translational processing of apoA-$I_{Tangier}$ which results in the formation of an isoform of apoA-$I_{Tangier}$ which is rapidly catabolized from plasma. The rapid catabolism of apoA-$I_{Tangier}$ results in reduced levels of HDL, the characteristic dyslipoproteinemia present in Tangier disease.

Objective:

4) Isolation and characterization of a fusion protein of cloned human apoB-100 in a λgt-11 expression vector.

Methods:

ApoB-100 has been cloned in a λgt-11 cDNA expression vector. The clones for apoB-100 were identified by screening with monospecific polyclonal apoB-100 antibodies. One of the clones, designated 24 λgt-B-100 was induced to synthesize the fusion protein containing β-galactosidase and the apoB-100 apolipoprotein by incubation with isothio-β-D-galactoside for 2 hrs at 37°C. Following induction the fusion protein was analyzed by $NaDodSO_4$ gel electrophoresis and immunoblot utilizing monospecific polyclonal and monoclonal antibodies to apoB-100. The size of the apoB-100 protein was determined by $NaDodSO_4$ gel electrophoresis.

Major Findings:

The fusion protein containing β-galactosidase (118,000 daltons) and the apoB-100 protein was expressed in high yield in the 24 λgtB-100 clone. The apparent molecular weight of the fusion protein was 180,000 daltons indicating the apoB-100 protein was approximately 60,000 daltons by $NaDodSO_4$ gel electrophoresis. Analysis of the fusion protein by immunoblot using a monospecific polyclonal apoB-100 antiserum revealed a postive band confirming that the fusion protein contained apoB-100. In addition, immunoblot of the fusion protein with monoclonal antibodies which blocked the apoB-100 LDL interaction with the LDL receptor were positve for the fusion protein. Thus results established that the fusion protein contained in 24 gtll B-100 clone contained the binding region for the LDL receptor.

Objective:

5) Solid phase synthesis of fragments of human apoC-II.

Methods Employed:

The total solid phase synthesis of apoC-II was performed utilizing a Beckman synthesizer model 990. The PAM resin was utilized in the synthesis since this resin has been effectively utilized in the synthesis of large proteins. The following groups were employed in the synthesis: Asp (OBzl), Glu (OBzl), Ser (Bzl), Thr (Bzl), Lys (Cl-2), Arg (TOS), His (TOS), and Trp (CHO).

The protein was removed from the resin by HF cleavage, and the formyl group removed from the tryptophanyl residue by 1 M piperidine in 8 M urea.

The synthetic apoC-II was purified to homogeneity by gel filtration, ion exchange chromatography, and high pressure liquid chromatography. The purity of synthetic apoC-II and apoC-II fragments was determined by polyacrylamide gel electrophoresis and Edman sequence analysis.

The ability of synthetic apoC-II to activate lipoprotein lipase was determined utilizing heparin-purified human lipoprotein lipase and a radiolabeled triolein substrate.

Major Findings:

Previous studies detailed in last years annual report described the solid phase synthesis of total apoC-II (residues 1-79). The synthetic apoC-II had complete biological activity when assayed in the lipoprotein lipase system. To gain further insight into the region of the apolipoprotein which is required for lipase activation, synthetic fragments of apoC-II containing residues 7-79 and 52 to 79 were prepared. The 7-79 fragment retained full biological activity, however the 52-79 fragment had little activity. These results were interpreted as indicating that the amino terminal half of apoC-II contains the structural domain which interacts with and activates lipoprotein lipase.

Significance to Biomedial Research and the Program of the Institute:

The isolation, characterization, and structural analysis of the plasma apolipoproteins and apolipoprotein isoforms is an ongoing series of studies which are designed to elucidate the physiological role and molecular mechanisms involved in the synthesis, transport, and metabolism of plasma lipoproteins in normal individuals and patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

The isolation, characterization, and structural analysis of the major plasma and lymph apolipoproteins will be continued, and an evaluation of the presence of plasma precursors of the apolipoproteins will be pursued. The enzymes which modualate the extracellular processing of the apolipoproteins will be analyzed. Of particular interest are the enzymes involved in the extracellular processing of apoA-I, apoA-II and apoC-II. In addition the enzyme(s) which function to decylated the circulating mature apolipoproteins will be investigated. Other possible types of post-translational modifications of the newly synthesized apolipoproteins will be studied, particularly with regard to carbohydrate and phosphorylated prosthetic groups. Studies will also be continued on the defect in Tangier disease. The enzyme(s) responsible for the conversion of the normal apoA-$I_{Tangier}$ to modified apoA-$I_{Tangier}$ which is rapidly catabolized will be pursued. These studies will add additional insights into the factors modulating the biosynthesis and catabolism of apoA-I

and HDL. The continued elucidation of the covalent structure and function of the plasma apolipoproteins will continue to be a prerequisite to our ultimate complete understanding of the molecular mechanisms involved in lipoprotein biosynthesis, transport, catabolism, and dyslipoproteinemias characterized by atherosclerosis.

Publications:

1. Bojanovski, D., Gregg, R.E., Ghiselli, G., Schaefer, E.J., and Brewer, H.B., Jr.: Human apolipoprotein A-I isoprotein metabolism: ProapoA-I conversion to mature apoA-I. J. Lipid Res. 26:185-193, 1985.

2. Brewer, H.B., Jr., Gregg, R.E., Bojanovski, D., Law, S.W., and Zech, L.A.: Genetic disorders of HDL apolipoprotein metabolism. In Miller, N.E. and Miller, G.J. (Eds.): High density lipoproteins: Clinical and metabolic aspects. Amsterdam, Elsevier Press, pp. 275-287, 1984.

3. Brewer, H.B., Jr., Sprecher, D.L., Gregg, R.E., and Hoeg, J.M.: Risk factors for the development of premature cardiovascular disease. In. Kritchevsky, D., Holmes, W.L. and Paoletti, R. (Eds.): Drugs Affecting lipid metabolism VIII vol. 183. New York, Plenum Press. pp. 27-36, 1985.

4. Hoeg, J.M., Brewer, H.B. Jr.: Human lipoprotein metabolism and the liver progress in liver disease. VIII H. Popper, ed. (in press) 1985.

5. Lackner, K.J., Edge, S.B., Gregg, R.E., Hoeg, J.M., and Brewer, H.B. Jr.: Isoforms of apoA-II in human plasma and thoracic duct lymph: Identification of proapoA-I and sialic acid containing isoforms. J. Biol. Chem. 260: 703-706, 1985.

6. Sprecher, D.L., Taam, L., and Brewer, H.B. Jr.: Two-dimensional electrophoresis of plasma apolipoproteins. Clin. Chem. 30:2084-2092, 1984.

7. Brewer, H.B. Jr., Ronan, R., Meng, M., and Bishop, C.: Isolation and characterization of apolipoproteins A-I, A-II, and A-IV. Methods in Enzymology. (in press), 1985.

8. Fairwell, T., Hospattankar, A.V., and Khan S.A.: Solid Phase Synthesis of human plasma apolipoprotein C-II and some of its fragment peptides. Proceedings of the Ninth American Peptide Symposium (in press), 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02011-10 MDB

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Molecular Properties of Lipoproteins and Apolipoproteins

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Prncipel Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	James C. Osborne, Jr., Ph.D.	Senior Investigator	MDB, NHLBI
Others:	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI
	Richard Gregg, M.D.	Senior Investigator	MDB, NHLBI
	Grace Huff, B.S.	Chemist	MDB, NHLBI
	Ellis Kempner, Ph.D.	Senior Investigator	LPB, NIADDK
	Nancy Lee, M.S.	Chemist	MDB, NHLBI
	Ramon R. Tate, Ph.D.	Senior Investigator	DCRT, NHLBI
	Loren Zech, M.D.	Senior Investigator	OD, NHLBI

COOPERATING UNITS *(if any)*
University of Umea, Sweden (G. Bengtsson, Ph.D. and T. Olivecrona, Ph.D.)

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.0	4.0	3.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

These projects are directed towards a greater understanding of the quaternary organization of plasma lipoproteins and of the function of the oligomeric species involved in the transport and metabolism of lipids in plasma. The apolipoprotein composition of plasma lipoproteins is viewed as the governing factor in directing lipoprotein metabolism. Specificity is believed to be related directly to apolipoprotein secondary, tertiary, and quaternary structure. A knowledge of the equilibrium constants and stoichiometry for the specific complexes formed in plasma by apolipoproteins has allowed us to develop a framework for evaluating the role of apolipoproteins in controlling lipid metabolism. These studies have been extended recently to include lipoprotein lipase and hepatic lipase, two enzymes responsible for triglyceride hydrolysis. We have shown that both active and inactive forms of lipoprotein lipase exist in solution. The active form is the dimer and dissociation results in irreversible inactivation. The active species of hepatic lipase is also the dimer. Radiation inactivation studies demonstrate that the minimal functional unit required for active human and bovine lipoprotein lipase and human hepatic lipase is the dimer. Although lipoprotein lipase and hepatic lipase have many similar properties, they are clearly different enzymes. Three new peptide modifiers of lipase activity have been identified. One of these peptides activates phospholipid hydrolysis by hepatic lipase while inhibiting corresponding triglyceride hydrolysis.

6 of 5

Objective:

Evaluation of the molecular weight of human lipoprotein and hepatic lipase by radiation inactivation.

The active species of bovine lipoprotein lipase is the dimer. Dissociation results in irreversible inactivation and is accompanied by a modest conformational change in the enzyme molecule. As summarized in last years annual report radiation inactivation studies of bovine lipoprotein lipase indicated that the molecular weight at low and high concentrations of enzyme was 72,000. Radiation inactivation of glycoproteins results in molecular weights that correspond only to the polypeptide portion of the macromolecule. Presumably this is due to the inability of a primary ionization in the carbohydrate moiety to disrupt the polypeptide backbone of enzymes. Bovine lipoprotein lipase contains 8.3% carbohydrate. Therefore, the 72,000 species observed by radiation inactivation corresponds to the dimer.

The results of radiation inactivation are thus consistent with the concept that the dimer is the minimal molecular unit required for active bovine lipoprotein lipase. Over the past year we have extended radiation inactivation studies to include human lipoprotein and hepatic lipases.

Methods Employed:

The assumptions used in the estimates of molecular weight by radiation inactivation are:

1) Primary ionizations are due to collisions between accelerated electrons and the macromolecule.
2) Primary ionizations are distributed randomly throughout the sample volume.
3) There are no indirect ionizations due to diffusion of free radicals generated from primary ionizations.
4) A single primary ionization (equivalent to the release of 1500 Kcal/mole in energy) is sufficient to disrupt an entire polypeptide chain.
5) There is no energy transfer between noncovalently bound protomers in a macromolecular complex.

With these assumptions target theory predicts that inactivation obeys the following equation:

$$A(D)/A_o = e^{-KD}$$

where

A_o = original activity
D_o = radiation dose
$A(D)$ = activity remaining after dose D
K = effective target volume

The effective target volume is obtained from the slope of a plot of ln A/A_o versus D (rads), and the molecular weight is obtained from a knowledge of the sample density (ρ) and Avogardro's number (N).

$$M = \rho N K$$

Major Findings:

A linear electron accelerator producing 13 MeV electrons at the Armed Forces Radiobiology Research Institute was used for radiation exposure. In order to minimize indirect effects, due to diffusion of free radicals from the location of the primary ionization, the samples were irradiated at -135°C. The buffer used was 0.5 M ammonium sulfate, 0.2 M sodium chloride, 0.001 M sodium azide, 0.01 M Tris/Cl, pH 7.4, which contained 5 mg/ml bovine serum albumin. As a control enzyme, glucose-6-phosphate dehydrogenase (0.3 unit/ml) was also included in each sample. The samples were sealed in 2-ml glass vials after previous rapid freezing in a slurry of dry ice and ethanol. After irradiation, the vials were opened and the samples were analyzed for lipase and glucose-6-phosphate dehydrogenase activity. Inactivation curves for glucose-6-phosphate dehydrogenase were monoexponential in all cases and corresponded to the expected molecular weight of 104,000. Experiments on lipoprotein and hepatic lipase were performed in the presence and absence of BSA, 1 mg/ml heparin and glycerol. Inactivation curves were independent of these changes in buffer composition. The curve for lipoprotein lipase was monoexponential in all cases and corresponded to a molecular weight of 104,650, a value substantially larger than the value of 72,000 found for bovine lipoprotein lipase. Initial experiments with hepatic lipase indicated a multiexponential decay of enzyme activity as a function of radiation dose. Plots of log A/A versus dose were convex with an initial slope corresponding to a target size of 55,000. Various control, experiments were performed and it was found that this result was due to the enzyme assay rather than the conditions of irradiation. The rate of triglyceride hydrolysis by hepatic lipase was dependent critically upon the fold dilution of stock active enzyme under assay conditions. Enzyme was appropriately diluted into buffer containing 1 mg/ml heparin prior to assay resulting in a monoexponential decay corresponding to a functional molecular weight of 83,700. Thus the functional molecular weights of human heaptic (83,700) and lipoprotein (104,650) lipases are higher than bovine lipoprotein lipase (72,000). Carbohydrate presumably does not contribute to values of molecular weight obtained by radiation inactivation. For the bovine enzyme active sedimentation equilibrium, where carbohydrate does contribute to the measured molecular weight indicates a molecular weight of 76,000 for the active species corresponding values for human lipoprotein lipase and human hepatic lipase are 92,300 and 73,100 respectively.

Objective:

Evaluation of the mechanism of regulation of enzymes involved in lipid metabolism: studies on peptide effectors of lipoprotein and hepatic lipase.

Human post heparin plasma contains two major lipases, lipoprotein lipase (LPL) and hepatic lipase (HL). LPL is associated with extrahepatic cells and is believed to play a major role in the hydrolysis of triglycerides in chylomicrons and VLDL. Triglyceride hydrolysis by LDL may be increased several fold in the presence of a specific activator, apolipoprotein C-II. Thus a deficiency in LPL or apoC-II may lead to the fasting chylomicronemia and mildly elevated VLDL which is characteristic of type I hyperlipoproteinemia. The metabolic role of HL is less well characterized. This enzyme is believed to be associated with hepatic tissue in vivo and has been postulated to play a role in HDL as well as VLDL phospholipid and triglyceride metabolism. The enzymic hydrolysis of triglycerides by HL can be increased several fold in vitro by the addition of apolipoprotein A-II. This activation has many of the characteristics of the apoC-II activation of LPL.

Other apolipoproteins have been shown to inhibit both lipoprotein and hepatic lipases in vitro. Thus apoA-I, C-I, C-II and C-III inhibit hepatic lipase and apoA-II, A-I, C-I and C-III inhibit lipoprotein lipase activity. Thus although LPL and HL have many common features, they are clearly different enzymes and can be physically as well as immunochemically separated from one another. Both lipoprotein lipase and hepatic lipase utilize triglyceride as well as phospholipid as substrates. Lipoprotein lipase is believed to function primarily as a triglyceride hydrolase where as hepatic lipase has been postulated to function primarily as a phospholipid hydrolase. We have now identified a series of peptides which inhibit the triglyceride hydrolase activity of hepatic and lipoprotein lipase and activate the phospholipid hydrolyase activity of the hepatic enzyme.

Methods Employed:

Circular dichroic measurement were performed using a Cary 61 spectropolarimeter. Data were obtained and processed automatically using a computer based data acquisition system designed by DCRT. Mean residence ellipticities (MRE) were calculated in the usual manner.

Major findings:

The peptides, pipinins I, II and III, were isolated from frog skin and sequenced in collaboration with John J. Pisano, NHLBI-LC. The sequence of Pipinin I is Phe-Leu-Pro-Ile-Ile-Ala-Gly-Val-Ala-Ala-Lys-Val-Phe-Pro-Lys-Ile-Phe-Cys-Ala-Ile-Ser-Lys-Lys-Cys. Pipinin II differs by a substitution of Ile for Val at position 8, and pipinin III differs from pipinin I by containing a serine residue at positions 7 and 14. All three peptides contain a 7 membered disulfide loop at the carboxy terminus. These peptides are among the most potent histamine-releasing compounds known. They are as potent as mastoparan in lysing erythrocytes and activate phospholipase A_2 in vitro.

In water pipinis I and II contain very little organized structure and resemble random coils. Increasing ionic strength results in minor increases

in structure (MRE = 4000 @ 220 nM). In a nonpolar environment, i.e. 80% methanol, both peptides gain substantial structure with the MRE at 220 nm increasing to approximately 12000. Pipinin III contains more structure in water (MRE = 6000 @ 220 nM) and increasing ionic strength results in MRE of 12000 at 220 nM. In 80% methanol the MRE of pipinin III increases to 20,000. Thus the minor differences in the sequences of these peptides results in major differences in secondary structure. All three peptides are quite flexable and assume numerous different shapes in order to accomodate a given environment.

Pipinin's I, II and III activate phospholipase A_2 with half maximal effects occuring at about 50 μmolar peptide. In this same concentration range, the pipinins inhibit the hydrolysis of triglyceride by lipoprotein lipase and hepatic lipase by approximately 80%. Increasing concentrations of peptide result in greater inhibition of triglyceride hydrolysis for both enzymes and parallel inhibition of phospholipid hydrolysis by lipoprotein lipase. In contrast, the phospholipase activity of hepatic lipase is increased by these higher concentrations of peptide. For instance, at 420 μmolar pipinin II the phospholipase activity of hepatic lipase is activated three fold whereas the corresponding triglyceride lipase activity is inhibited by 90%. These results raise the intriguing possibility that lipase specificity towards triglyceride and phospholipid may be controlled in vitro and may lead to a fuller understanding of the in vivo roles of lipoprotein and hepatic lipases.

Significance to Biomedical Research and the Program of the Institute:

These investigations are directed towards a greater understanding of the quaternary organization and function of the palsma lipoproteins. The apolipoprotein and lipid composition of plasma lipoproteins is related directly to the concentration and composition of other components of plasma, including other plasma lipoproteins. Enzymic hydrolysis of lipids in plasma is mediated through apolipoprotein effectors which distribute among plasma lipoproteins according to the laws of mass action. A quantitative knowledge of these types of interactions is fundamental to our understanding of lipid transport and metabolism in normal individuals and in patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

Investigations concerning the molecular properties of apolipoproteins and enzymes involved in lipid metabolism shall be continued. A major emphasis shall be placed on evaluation of the role of lipoprotein lipase and hepatic lipase in plasma lipoprotein metabolism. Studies on the apolipoprotein and peptide effectors of these enzymes shall be continued with particular emphasis on controlling the specificity towards triglyceride and phospholipid hydrolysis.

Publications

1. Hoeg, J.M., Demosky, S.J., Edge, S.B., Gregg, R.E., Osborne, J.C., Jr. and Brewer, H.B., Jr.: Characterization of a human hepatic receptor for high density lipoproteins. Arteriosclerosis. 5, 228-237, 1985.

2. Osborne, J.C., Jr. Lee, N.S. and Powell, G.M.: Solution properties of apolipoproteins: Methods in Enzymology, in press.

3. Osborne, J.C., Jr. : Delipidation of plasma lipoproteins: Methods in Enzymology in press.

4. Osborne, J.C., Jr., Bengtsson, G., Lee, N.S. and Olivercrona, T.: Studies on inactivation of lipoprotein lipase: Role of the dimer to monomer dissociation. Biochemistry, in press.

5. Olivercrona, T., Bengtsson-Olivercrona, G., Osborne, J.C., Jr. and Kempner, E.S.: Molecular size of bovine lipoprotein lipase as determined by radiation inactivation. J. Biol. Chem. 260, 6888-6891, 1985.

6. Moss, J., West, R.E., Jr., Osborne, J.C., Jr. and Levine, R.L.: Characterization of NAD: Arginine mono-ADP-ribosyltransferases in turkey erythrocytes: Determinants of substrate specificity. In Proceedings of the Seventh International Symposium on ADP-Ribosylation Reductions, Vitznau, Switzerland, September 1984, in press.

7. Saxena, A., Hensley, P., Osborne, J.C., Jr. and Fleming, P.J.: The pH-dependent subunit dissociation o dopamine beta-hydroxylase. J. Biol. Chem. 260: 3386-3392, 1985.

8. Osborne, J.C., Jr., Stanley, S.J. and Moss, J.: Kinetic Mechanisms of two NAD: Arginine ADP-Ribosyltransferases: the soluble, salt-stimulated transferease from turkey erythrocytes and choleragen, a toxin from Vibrio Cholerae: Biochemistry, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02012-10 MDB

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Hepatic 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Zafarul H. Beg, Ph.D.	Research Chemist	MDB, NHLBI
Others:	J.A. Stonik	Chemist	MDB, NHLBI
	H.B. Brewer, Jr., M.D.	Chief	MDB, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously demonstrated that rat and human liver HMG-CoA reductase activity is modulated *in vitro* and *in vivo* in a bicyclic cascade system involving reversible phosphorylation of both reductase kinase and HMG-CoA reductase. Recently, we have also reported that enzyme activities of both native (M_r 100,000) and protease-cleaved soluble HMG-CoA reductase (M_r 53,000) from rat liver are modulated by a Ca^{2+}-activated and phospholipid-dependent protein kinase C mediated phosphorylation.

During the past year we have purified and characterized a low molecular weight (M_r 120,000) Ca^{2+}, Calmodulin-dependent protein kinase (CMK) from rat brain cytosol. This CMK is different from other CMK both in terms of holoenzyme molecular weight and substrate specifity. The purified CMK was able to phosphorylate both insoluble microsomal (M_r 100,000) and purified soluble (M_r 53,000) HMG-CoA reductase. Maximal phosphorylation of HMG-CoA reductase was associated with the incorporation of 1 mol of phosphate/mol of enzyme (M_r 100,000). Dephosphorylation of ^{32}P-HMG-CoA reductase was associated with loss of radioactivity and reactivation of enzyme activity.

The identification of the CMK system in addition to the reductase kinase and C Kinase systems for the modulation of the enzymic activity of HMG-CoA reductase may provide new insights into the molecular mechanisms involved in the regulation of cholesterol biosynthesis.

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Project Description:

Objectives: The current research of this laboratory is related to the purification and characterization of a new protein kinase, Ca^{2+}, calmodulin dependent kinase and its role in the phosphorylation of native (M_r 100,000) and soluble HMG-CoA reductase (M_r 53,000).

Methods Employed:

1) Purification of Ca^{2+}, calmodulin-dependent kinase from rat brain: Forebrains (36g) from 30 female Sprague-Dawley rats (160g) were removed and homogenized with 182 ml of 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 50 mM 2-mercaptoethanol, 10% glycerol, 0.5 mM RMSF, 0.1 mMTPCK, 0.1 mM TLCK and 0.05 mM leupeptin, followed by centrifuged for 10 min at 16,000 x g. The supernatant was recentrifuged at 240,000 x g for 30 min. The resultant brain cytosol (162 ml) was applied on a DEAE (DE 52) cellulose column (4.4 x 8 cm), which was previously equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 50 mM 2-mercaptoethanol, 10% glycerol, 0.5 mM PMSF, 1 mg/liter leupetin, 0.1 mM TLCK and 0.1 mMTPCK). The column was washed with 200 ml of buffer A, 300 ml of buffer B (same as buffer A except 1 mM EDTA and 1mM EGTA added), and eluted with a gradient of 450 ml of buffer B and 400 mM NaCl in buffer B (450 ml). Fractions (12 ml) were collected and assayed for protein and conductivity. Calmodulin-dependent protein kinase activity in each fraction was determined by utilizing purified HMG-CoA reductase as substrate. Fractions containing calmodulin-dependent kinase activity were pooled and fractionated with ammonium sulfate (40%), centrifuged and the pellet dissolved in 65 ml of buffer C (40 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, 1 mg/liter leupeptin, 10% glycerol, and 0.2 mM $CaCl_2$) containing 0.2 m NaCl. The supernatant (65 ml) was applied to a column (1x8 cm) of agarose-calmodulin previously equilibrated with buffer C. After washing the column sequentially with 200 ml of buffer C containing 2M NaCl followed by 2.0 ml of buffer C containing 0.2 M NaCl, kinase activities were eluted with 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM EGTA, 10% glycerol, and 10 mM 2-mercaptoethanol. Two ml fractions were collected, analyzed for HMG-CoA reductase kinase activity stimulated by calmodulin and Ca^{2+}. Fractions containing kinase activity were pooled, fractionated with ammonium sulfate (40% saturation), and the pellet dissolved in 1.6 ml of buffer D (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.2 M NaCl, 50 mM 2-mercaptoethanol, 0.5 mM PMSF, 0.1 mM TPCK, 0.1 mM TLCK, 1 mg/liter leupeptin, and 10% glycerol). The supernatant was applied to a column (2.2 x 185 cm) of Fractogel TSK HW-55 (F) equilibrated with buffer D. The eluted fractions (2 ml) were analyzed for protein and calmodulin-stimulated HMG-CoA reductase kinase activity. The enzyme was stored frozen at -70°C.

2) Isolation and Assay of Microsomal and Purified HMGR Activity:

Microsomes isolated from livers of rats fed 3% cholestyramine for 5 days and specified at the peak of diurnal rhythm were used for solubilization and purification of homogeneous HMGR. This method of isolation involves the purification of the 53,000 molecular weight form of HMGR followed by a

protease mediated solubilization of microsomal HMGR. Antibodies to purified rat liver HMGR were prepared in New Zealand rabbits by subcutaneous injections of a total of 480 ug of protein per rabbit. The first two injections (1st and 3rd weeks) were in complete Freund's adjuvant; all subsequent injections were given at 12 and 17 week intervals in incomplete Freund's adjuvant. Blood was withdrawn 18, 19, 20, and 21 weeks following the initial injection and analyzed for antibody titer. The native form of microsomal HMGR (M_r 100,000) was isolated and the microsomal pellet containing native insoluble membrane bound HMGR was suspended in 40 mM MOPS, (pH 6.5), 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 50 uM PMSF, 50 uM leupeptin, and 10% glycerol (buffer A). The microsomal suspension was heated at 50°C for 15 min. This technique selectively inactivates the heat labile microsomal reductase kinase whereas HMGR activity remains unaffected. An aliquot of heat treated microsomal HMGR was solubilized by homogenizing in 1% lubrol followed by centrifugation (100,000 xg, 60 min). The supernatant was fractionated with 60% ammonium sulfate and the precipitate dissolved as well as desalted in buffer A. Microsomal and solubilized HMGR were utilized for the inactivation and phosphorylation of the native high molecular form (M_r 100,000) of HMGR with protein kinase C. Enzymic activity of microsomal, solubilized, and purified HMGR were determined as previously described in other annual reports.

3) Assay Ca^{2+}, Calmodulin - Dependent Kinase Activity and HMG-CoA Reductase Phosphorylation: Enzyme fractions were assayed in a total volume of 25 ml containing 25 mM MOPS (pH 6.5), 1 mM Ca^{2+}, 5 uM calmodulin, 6mM MG^{2+}, 0.1 mg/ml purified HMG-CoA reductase (unless stated otherwise), and 0.05 mM [^{32}P] ATP (specific activity 1700-3400 cpm/pmol). Control incubations lacked Ca^{2+} and calmodulin and contained 5 mM EGTA. Tubes were incubated at 30°C for 10 min or as indicated in each experiment. At the end of the incubation time, duplicate 5 ul aliquots were spotted onto Whatman 3 MM filters discs which had previously been treated with 50 ul of 20% trichloroacetic acid containing 1 mM ATP and 5 mM sodium pyrophosphate. The filter paper discs were washed for 30 min in 20% trichloroacetic acid containing 1 mM ATP and 5 mM sodium pyrophosphate, and then three times for 30 min each in 5% trichloroacetic acid. After final wash, discs were dried in methanol followed by diethylether, and counted in 5 ml of hydrofluor (National Diagnostic). One unit of calmodulin-dependent kinase is defined as the activity which transfers 1 nmol of phosphate from [^{32}P] ATP to purified HMG-CoA reductase or other substrates per min under the assay conditions described above.

For analysis of [^{32}P]-HMG-CoA reductase, aliquots were added to tubes containing 1% SDS, 2 mM DTT, 30% glycerol, 0.002% bromophenol blue, and heated in boiling water for 4 min. The proteins were separated by one-dimensional SDS-PAGE, stained with Coomassie blue, dried, and exposed on XAR-5 (Kodak) film at - 120°C. Dried gels or autoradiograms were quantitated by densitometric scanning using a Beckman DU-8 spectrophotometer.

Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was performed according to the method of Lemmli with 1.5 mm thick slab gels and the indicated percentage of (acrylamide acrylamide/bisacrylamide ratio of 30/0.8). The molecular weights of the separated subunits were determined by comparison in SDS-PAGE of known molecular weight standards.

4) Autophosphorylation of calmodulin-dependent protein kinases: Autophosphorylation of different molecular forms of calmodulin-dependent protein kinases was performed as outlined above for the assay of calmodulin-dependent kinase activity except that HMG-CoA reductase was omitted from the reaction mixture. Aliquots of reaction mixture were removed and mixed with SDS-PAGE buffer (as described above) to give a final concentration of 1% SDS and heated for 4 min at 100°C. The samples were analyzed by SDS-PAGE.

5) Calmodulin Binding:

^{125}I-calmodulin binding to peptides resolved in SDS-PAGE was performed according to the method of Carlin et al. Gels after SDS-PAGE were fixed in 25% isopropyl alcohol and 10% acetic acid for 12 hr, followed by incubation for 12 hr. in 50 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 2 mM Ca^{2+} (buffer G). Gels were then washed for 2 hr with buffer G containing 1 mg/ml of bovine serum albumin. The gels were incubated for 12 hr with 50 ml of buffer G containing 2 uCi of ^{125}I-calmodulin. The gels were finally washed for 24 hr in buffer G with at least six changes, stained with Coomassie blue, dried under vacuum, and exposed to x-ray film at -20°C.

6) Immunoprecipitation and Immunoblotting:

Aliguots of phosphorylated native microsomal or lubrol-solubilized ^{32}P-HMG-CoA reductase were incubated for 30 min at room temperature in 10 mM phosphate buffer, pH 7.5, containing 1% lubrol, 0.5% deoxycholate, 0.1% SDS, 0.1 M NaCl, 5 mM EDTA, 5mM EGTA and 200 uM leupeptin (buffer H). Each tube was precleaved by incubation for 30 min with 10 ul of 10% Pansorbin (v/v in buffer H) and centrifuged. Normal rabbit or anti-HMG-CoA reductase serum was then added to the clear supernatant. After 20 hrs at 4°C, 100 ul of 10% Pansorbin was added and the sample incubated at room temperature for 60 min. After centrifugation, immunoprecipitates were washed three time with buffer H and then incubated at room temperature for 45 min with 50-75 ul of buffer I (62.5 mM Tris-HCl, pH 6.5, 15% SDS and 10% B-mercaptoethanol). The pansorbin was pelleted and the supernatant was transfer to tubes containing glycerol, bromophenol blue, and solid urea to a final concentration of 50%, 0.05%, and 8.0 M, respectively. The samples were incubated at 37°C for 20 min and analyzed by SDS-PAGE (7.5%) according to Laemmli except both running and stacking gels contained 4 M urea. All gels were dried and processed for autoradiography.

Immunoblotting was carried out as described in the immunoblotting GAR-HRP assay kit instructions from the Bio-Rad laboratories. Microsomal or solubilized proteins were separated by SDS-PAGE and electrophoretically

tranferred to nitrocellulose sheets and detected by utilizing HMG-CoA reductase antiserum.

Major Findings:

Isolation and Characterization of Ca^{2+}, Calmodulin - Dependent HMG-CoA Reductase Kinase:

Earlier reports indicate that brain Ca^{2+}, calmodulin-dependent kinases are very unstable. The instability of kinase activity has been attributed to various proteases cleavage and/or loss of activity due to modification of the catalytic site by oxidation, etc. To preserve kinase activity, rat brains must be homogenized in the presence of protease inhibitors and purification must proceed rapidly. The cytosol from rat brains was applied to a DEAE cellulose column and eluted with a salt gradient. The fractions containing calmodulin-dependent kinase activity were pooled, dissolved in buffer C and applied to a calmodulin affinity resin. The calmodulin-affinity purified enzyme was fractionated with ammonium sulfate (40% saturation), dissolved in buffer D, and further purified by fractionation on Fractogel TSK HW-55. Two peaks containing calmodulin stimulated kinase activity were separated. Peak I and Peak II fractions were assayed for calmodulin-dependent kinase activity utilizing both purified HMG-CoA reductase and histone H_1 as substrates. Both substrates were phosphorylated in a similar manner. Peak II revealed higher calmodulin-stimulated HMG-CoA reductase kinase activity and lower kinase autophosphorylation and was employed in the current studies.

The purification scheme outlined above has allowed the extensive purification of a enzymically stable calmodulin-dependent protein kinase. Calmodulin stimulated HMG-CoA reductase kinase activity was readily detectable at different stages of purification including the cytosol. However, in crude fractions reductase kinase activity (in the absence of Ca^{2+} and calmodulin) was present.

Molecular Weight of Holoenzyme and Subunit Composition:

From the calibration of the Fractogel column with standard proteins of known molecular it is estimated that the peak 2 kinase had a molecular weight of 120,000. Consistent with published reports peak 1 holoenzyme had a molecular weight of 600,000. Analysis of Fractogel purified peak 2 by HPLC also revealed an apparent molecular weight of 120,000.

On SDS-gel electrophoresis, the purified enzyme contained two major peptide components of approximately 54,000 and 62,000 daltons, designated as L and B, respectively. The B peptide contained both a lower M_r 60,000 B polypeptide in addition to the upper B subunit of M_r 62,000. The M_r 54,000, 60,000 and 62,000 proteins constituted 85-90% of the total protein mass. A M_r 46,000 polypeptide was the only other contaminant detected in the peak II purified preparation, whereas peak I kinase (M_r 600,000) was electrophoretically homogeneous. The inability of the M_r 46,000 component to bind calmodulin or to be phosphorylated suggested that it was not a

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dissociated product of the higher molecular weight subunits. Immunoblotting of peak I and peak II enzyme fractions with a calmodulin-dependent phosphatase antiserum revealed the absence of calmodulin-dependent phosphatase in the purified fractions.

Both the L and the two B subunits (B/B') bound calmodulin in denaturing gels by the gel overlay method. Calmodulin binding to L and B/B' subunits was significantly reduced following washing with an EGTA-containing buffer. The L subunit has a higher affinity for calmodulin than the B/B' subunits, although the Coomassie stained band of B/B' subunits were more intense. Whether or not differntial binding of calmodulin to the proteins in denaturing gels reflects the actual affinity of the native enzyme for calmodulin remains to be determined.

Effect of pH on Calmodulin-dependent kinase-Mediated Phosphorylation of HMG-CoA Reductase:

In order to determine optimal pH for HMG-CoA reductase phosphorylation, effect of pH in the range of 5.0 to 9.0 units was examined. A broad pH optimum with a maximum at pH 6.5 was observed. The inhibition of enzymic activity of HMG-CoA reductase was also maximal at pH 6.5. The high molecular weight calmodulin-dependent kinase (M_r 600,000) isolated from brain and liver revealed a pH optima of 7.0-7.5 utilizing different substrates.

Substrate Specifity:

Calmodulin-dependent kinase phosphorylated a variety of substrates. The most significant substrate other than HMG-CoA reductase was lysine rich histone (S-III). Protamine, phosvitin, casein, muscle glycogen synthase, and arginine rich histone (VIII-S) were poorly phosphorylated. Muscle phosphorylase b and muscle myosin light chains showed little kinase activity. No phosphorylation of purified HMG-CoA reductase was observed with protein kinase in the absence of Ca^{2+} and calmodulin, suggesting the absence of reductase kinase activity in the purified preparation. Similarly cAMP-dependent protein kinase activity was not detected in the Fractogel-purified enzyme.

Phosphorylation of Native and Purified HMG-CoA Reductase

Incubation of purified soluble HMG-CoA reductase with calmodulin-dependent kinase, Ca^{2+}, calmodulin and ATP-Mg was associated with a time-dependent inactivation of HMG-CoA reductase. Treatment of inactivated HMG-CoA reductase with phosphatase revealed a time-dependent reactivation of enzyme activity. The phosphatase-mediated reactivation of inactivated HMG-CoA reductase was blocked by NaF. The calmodulin-dependent kinase catalyzed inactivation was associated with the phosphorylation of purified HMG-CoA reductase. The results demonstrate that enzyme inactivation was directly correlated with the incorporation of ^{32}P phosphate. 0.69 mol of phosphate was incorporated per mol of native HMG-CoA reductase (M_r 100,000) with a 72% concomittant inactivation of purified HMG-CoA reductase enzymic

activity. Maximal phosphorylation of purified HMG-CoA reductase by calmodulin stimulated kinase revealed a stoichiometry of approximately 1 mol of phosphate/mol of M_r 100,000 enzyme. Autoradiography of purified phosphorylated HMG-CoA reductase following $NaDodSO_4$-PAGE revealed a radioactive band corresponding to the 53,000 molecular fragment of the purified enzyme.

Phosphorylation of HMG-CoA reductase in the presence of calmodulin plus EGTA, Ca^{2+} alone, and in the absence of Ca^{2+} and calmodulin (plus EGTA), failed to show radioactivity in the HMG-CoA reductase band. Dephosphorylation of ^{32}P-HMG-CoA reductase with hepatic phosphatase was associated with the loss of radioactivity in the HMG-CoA reductase band in the autoradiogram. Control samples containing NaF-inactivated phosphatase were not associated with any decrease in the ^{32}P radioactivity.

The calmodulin-dependent protein kinase was able to inactivate the enzymic activity of the native microsomal HMG-CoA reductase (M_r 100,000) when incubated in the presence of Ca^{2+}, calmodulin and ATP-Mg. The inactivated (phosphorylated) HMG-CoA reductase enzyme from microsomal preparations containing the 100,000 molecular form was also reactivated (dephosphorylated) by purified phosphatase. Phorphorylation of the native microsomal form of HMG-CoA reductase was confirmed by determination of ^{32}P radioactivity in the native enzyme following immunoprecipitation with HMG-CoA reductase antiserum and analysis by $NaDodSO_4$-Urea-gel electrophoresis. Samples incubated with normal rabbit serum or control samples containing no Ca^{2+} and calmodulin failed to incorporate radioactivity in the area corresponding to the 100,000 molecular form of native HMG-CoA reductase.

Significance to Biomedical Research and the Program of the Institute:

Based on the results described in this report and our previous *in vitro* and *in vivo* studies we now propose that HMG-CoA reductase activity is modulated by three separate kinase systems. The first system involves the bicyclic cascade system involving reductase kinase and reductase kinase kinase. The second system involves protein kinase C. Increased phosphorylation of HMG-CoA reductase by phorbol ester, PMA, suggests that the protein kinase C - mediated phosphorylation may be physiologically important mechanism *in vivo*. A third mechanism for the modulation of HMG-CoA reductase detailed in this report involves a Ca^{2+}, calmodulin-dependent kinase. The elucidation of the modes of control of HMG-CoA reductase will allow a detailed analysis of the factors involved in the cellular regulation of cholesterol metabolism in normal subjects and patients with atherosclerosis.

Proposed Course:

A systematic investigation of the third kinase system mediated by Ca^{2+}, calmodulin-dependent kinase, will be continued *in vitro* and *in vivo*. In

addition studies will be performed to determine which physiological systems regulate the enzymic activity of HMG-CoA reducatase by modulating the activity of the three separate kinase systems.

Publications:

1. Beg, Z.H., Stonik, J.A., and Brewer, H.B., Jr.: Human hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase. Evidence for the regulation of enzymic activity by a bicyclic phosphorylation cascade. Biochem. Biophys. Res. Commun. 119:488-498, 1984.

2. Beg, Z.H., Stonik, J.A., and Brewer, H.B., Jr.: In vivo modulation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase, reductase kinase and reductase kinase kinase by mevalonolactone. Proc. Natl. Acad. of Sci. USA,*!:7293-7297, 1984

3. Beg, Z.H., Stonik, J.A., and Brewer, H.B., Jr.: Phosphorylation of Hepatic HMG-CoA Reductase and Modulation of It's Enzymic Activity by Calcium -activated and phospholipid-dependent Protein Kinase. J. Biol. Chem. 260:1682-1687, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02019-07 MDB

PERIOD COVERED: October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*: Metabolism of Lipoprotein and Apolipoproteins in Humans

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigetor.) (Neme, title, leboratory, and institute affiliation)*

PI:	Richard E. Gregg, M.D.	Senior Investigator	MDB, NHLBI
Others:	Loren A. Zech, M.D.	Senior Investigator	MDB, NHLBI
	Paola Roma	Visiting Fellow	MDB, NHLBI
	Diana Wilson	Chemist	MDB, NHLBI
	Debrough Stark	Chemist	MDB, NHLBI
	Lila Taam	Chemist	MDB, NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI

COOPERATING UNITS *(if eny)*:

LAB/BRANCH: Molecular Disease Branch

SECTION: Peptide Chemistry

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	1.5	3

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
- [X] (a1) Minors
- [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the spece provided.)*

The ELISA assay has been automated and the apoA-I ELISA assay is presently being validated. Apolipoprotein E is a polymorphic protein with 3 common forms, $apoE_2$, E_3, and E_4, with $apoE_2$ being catabolized the slowest and $apoE_4$ the fastest in humans. $ApoE_2$ has two reactive cysteines while $apoE_4$ has these cysteines replaced with arginines. The slow metabolism of $apoE_2$ was determined to be due predominantly to the altered conformation of the protein resulting from the substitution of cysteine for the arginine with only a small portion of the slower catabolism of the $apoE_2$ being secondary to the slow catabolism of the disulfides dimers. ApoE is a glycoprotein that is present in plasma as di, mono, and asialo-apoE. When disialo-apoE was injected into subjects, there was little conversion to the mono or asialo forms indicating that either apoE is secreted with carbohydrate heterogeneity or that the apoE is modified following secretion but before it enters the plasma apoE pool. Tangier disease patients have low levels of HDL and apoA-I resulting from rapid catabolism of HDL. Two forms of apoA-I were isolated from Tangier subjects, one that co-migrated with normal apoA-I and one that was slightly shifted in molecular weight and isoelectric point. The normal form had a normal catabolic rate in kinetic studies while the shifted form was rapidly catabolized. This is consistent with the defect in Tangier disease being a post-translational modification of apoA-I in the circulation leading to rapid catabolism. Abetalipoproteinemia has been considered to be secondary to the absence of apoB synthesis. By utilizing sensitive immunological methods for the detection of apoB, it could be detected in plasma and in hepatocytes from abetalipoproteinemia subjects. This indicates that in at least some affected kindreds the defect is a post-translational abnormality that leads to the inability to secrete apoB.

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Project Description

The current research of this laboratory is the following:

Objective:

1) To develop the methodology for the measurement of apolipoproteins by enzyme linked immunoassay techniques.

Methods Employed:

In a competitive solid phase enzyme linked immunosorbent assay the antigen is bound to polystyrene microtite plate wells followed by the addition sample or standard and an antibody against the substance to be quantitated. The wells are washed and the amount of antibody bound to the plates is assayed using an alkaline phosphatase conjugated anti-IgG antibody. From this, the concentration of analyte in the assay sample can be determined.

Major Findings:

The ability to perform enzyme lined immunosorbent assays (ELISA) has been automated. The samples are automatically diluted and pipetted into 96 well microtiter plates, the wells are washed with a 96 port microtiter plate washer, the development of color is quantitated with an automated spectrophotometer, and the data is directly transferred from the spectrophotometer to a computer for data analysis. In addition to the apoB ELISA previously developed, an apoA-I ELISA currently being validated and an apoE ELISA is in the early stages of development.

Objective:

2) To study the metabolism of apolipoprotein E in normal and dyslipoproteinemic subjects.

Methods Employed:

ApoE kinetics were studied by isolating apoE by ultracentrifugation and column chromatography, radioiodinating with iodine monochloride, reassociating it with lipoproteins, and injecting it intravenously into the study subjects. Multiple timed plasma samples were obtained, the lipoproteins separated by ultracentrifugation, and the rate of catabolism determined from the radioactive decay curve by computer assisted multiexponential curve fitting.

Major Findings:

ApoE is found in plasma primarily on VLDL and HDL and is important in modulating the catabolism of remnants of triglyceride rich lipoprotein particles. ApoE is a polymorphic protein in the population with common

isoforms, designated as $apoE_2$, $apoE_3$, and $apoE_4$, with $apoE_3$ being the most common form in the population and is considered to be the normal isoform. An individual can be either heterozygous or homozygous for any of these apoE forms. $ApoE_2$ is associated with lower than normal levels of LDL cholesterol while $apoE_4$ is associated with elevated LDL cholesterol levels. I have previously determined that, compared to $apoE_3$, $apoE_2$ is catabolized more slowly than $apoE_3$ in humans while $apoE_4$ is catabolized more rapidly. The only differences between $apoE_2$ and $apoE_4$ are at amino acid positions 112 and 158 with $apoE_2$ having cysteines at both positions and $apoE_4$ having arginines. The cysteines are reactive and have been shown to form disulfides with apoE and with other plasma proteins that contain free sulfhydryl moeities. In order to determine if the difference in the catabolism between $apoE_2$ and $apoE_4$ was due to the presence of the positively charged arginines in $apoE_4$ or if it was due to the lack of cysteines and the ability to form disulfides, the following study was performed. $ApoE_2$ was modified with the addition of either methyl groups or aminoethyl groups to the free sulfhydryls in order to block the sulfhydryls in the first case and to form a positively charged arginine analogue in the second. The in vivo metabolic kinetics of these modified $apoE_2$ proteins were than compared to $apoE_2$ and $apoE_4$. The aminoethyl $apoE_2$ was metabolized at the same rate as $apoE_4$ while the methyl $apoE_2$ was metabolized at an intermediate rate between $apoE_2$ and $apoE_4$. These results are consistent with both the loss of the ability to form disulfides and the addition of the positive charges being responsible for the rapid catabolism of $apoE_4$.

ApoE is a glycoprotein with the circulating plasma form being predominately asialo apoE with small amounts of mono and disialo apoE. In order to determine the rate of conversion of the sialo forms of apoE to the asialo forms, disialo and asialo isoforms of apoE were isolated and their in vivo metabolism determined. Both forms of apoE were metabolized at approximately the same rate and there was little or no conversion of the disialo to the asialo form. Therefore, either apoE is secreted as both sialo and asialo forms, or the reinjected apoE does not have access to the site of sialic acid cleavage, i.e. the sialic acid may be removed from newly secreted apoE on nascent lipoprotein particles or in the lymphatic space before it reaches the systemic circulation.

Objective:

3) To study the metabolism of normal and variant forms of apolipoprotein A-I in normal and dyslipoproteinemic subjects.

Methods Employed:

The methods for performing apoA-I kinetic studies have been modified and extended. ApoA-I and isoforms of apoA-I were isolated from plasma and lymph by ultracentrifugation, column chromatography, and preparative sodium dodecyl sulfate and isoelectrofocusing gel electrophoresis. The isolated isoforms of apoA-I were iodinated by the iodine monochloride method, injected into study subjects, and multiple timed samples of plasma obtained.

The radiolabeled isoforms of apoA-I were than isolated by ultracentrifugation and preparative isoelectrofocusing; and the rate of catabolism of radiolabeled apoA-I isoforms from plasma was determined by computer curve fitting.

Major Findings:

Tangier disease is characterized by having extremely low levels of circulating HDL, apoA-I, and apoA-II with rapid catabolism of HDL and its components. Two forms of apoA-I can be isolated from Tangier subjects, one with a normal molecular weight and isoelectric point and one with a slight shift of both the molecular weight and isoelectric point. These two forms of apoA-I were isolated from a Tangier patient and their in vivo metabolism quantitated. The normal form of Tangier apoA-I had a normal catabolic rate in a control subject while the shifted form had a very rapid catabolic rate. An extremely small amount of the shifted form could also be isolated from normal plasma, and it had a similarly rapid catabolic rate. The understanding of the process that leads to the formation of this shifted form of apoA-I should give insights into the basic molecular defect in Tangier Disease, and may give information as to the mechanism by which apoA-I is catabolized in normal individuals.

Objective:

4) To investigate the molecular defect in abetalipoproteinemic subjects that leads to the extremely low to absent circulating plasma apoB concentrations.

Methods Employed:

The presence of apoB on lipoprotein particles is being determined by both immunoblot methods and by a sensitive ELISA assay following an approximately 500 fold concentration of the lipoproteins by ultracentrifugation. Immunohistochemistry methods are being employed to determine the presence of apoB in tissues, while both classical and molecular genetic methods are being utilized to determine the genetics of the hereditary defect in abetalipoproteinemic subjects.

Major Findings:

Following a 500 fold concentration of lipoproteins by ultracentrifugation, apoB could be detected in the plasma of three different kindreds of abetalipoproteinemic subjects. The apoB was of a higher apparent molecular weight by SDS gel electrophoresis in all three kindreds. ApoB could also be detected in hepatocytes from an abetalipoproteinemic individual by immunohistochemistry and light microscopy utilizing both monoclonal and polyclonal immunoglobulins. In a large family with abetalipoproteinemia, the disease did not co-segregate with an enzyme polymorphism near the known location of apoB on chromosome 2, indicating that the chromosomal location of the genetic defect in abetalipoproteinia is

not closely linked to this enzyme polymorphism. In conjunction with the finding of the near normal apoB mRNA levels in abetalipoproteinemic hepatocytes (see annual report of Dr. Simon Law), these results indicate that in at least some abetalipoproteinemic individuals, the primary genetic defect is in the post-translational modification of the protein that results in an inability to secrete apoB and apoB containing lipoproteins. The molecular defect could be in the primary structure of apoB leading to an altered post-translational modification of it, or in one of the enzymes necessary for the modification of the newly synthesized nascent apoB protein.

Significance to the Biomedical Research Program of the Institute

Lipoproteins are very important in initiating and modulating the atherosclerotic process. Apolipoproteins are central to the control of lipid and lipoprotein metabolism and by understanding the metabolism of apolipoproteins new insights are gained into the control of the development of atherosclerotic vascular disease. There are a number of dyslipoproteinemic states in which there is a known abnormality in an apolipoprotein or apolipoprotein receptor. These include type III hyperlipoproteinemia, apoE absence, familial hypercholesterolemia, Tangier disease and abetalipoproteinemia. By studying these dyslipoproteinemic states in which nature has introduced a known specific pertubation, one can gain a more complete understanding of lipoprotein metabolism in these patients as well as normal subjects. This will allow a more complete and rational approach to the therapy of diseases characterized by dyslipoproteinemia.

Proposed Course:

It is proposed to extend these studies in the following ways:

1) The development of an enzyme linked immunoassay for apoA-I will be completed. Immunoassays for other apolipoproteins will also be converted to the enzyme linked immunoassay method because of inherent advantages of this type of assay compared to the present methods being employed.

2) The investigation of the role of apoE in lipoprotein metabolism will be continued. Macrophages from an individual with apoE deficiency will be cultured, and these macrophages that are unable to synthesize and secrete apoE will be characterized as will the ability of apoE deficient lipoproteins to bind to lipoprotein receptors. Specifically, the ability of lipoproteins to bind to the macrophages and the removal of cholesterol from these macrophages when they are cholesterol loaded will be quantitated. The molecular defect associated with apoE deficiency will also be determined, in collaboration with Dr. Simon Law, by sequencing the apoE gene from an affected individual. In addition, the affect of apoE on modulating lipoprotein lipase and hepatic lipase will be investigated.

3) The studies on Tangier disease will continue. Most of the evidence at the present time indicates that the defect in Tangier disease is

a rapid catabolism of apoA-I and apoA-II which have normal primary amino acid sequences. This could be due to an intravascular alteration of these proteins or to an abnormal receptor interaction that leads to their rapid catabolism. These possibilities will be evaluated utilizing cells from Tangier subjects in tissue culture. In addition, the kinetics of apoA-I metabolism in subjects with low apoA-I and HDL levels and a restriction endonuclease fragment length polymorphism linked to the apoA-I gene will be determined.

4) The role of apoB in lipoprotein metabolism will continue to be investigated. The role of the LDL receptor in regulating synthetic and catabolic rates of the different classes of lipoproteins will be determined by quantitating the kinetics of apoB metabolism in VLDL, IDL, and LDL in receptor negative familial hypercholesterolemic homozygotes. In addition, the defects in the different types of abetalipoproteinemia will be further analyzed by enzymatically modifying the small amount of apoB in their plasma to determine how it is different from normal apoB, by determining the subcellular localization of the apoB present in hepatocytes from abetalipoproteinemic subjects utilizing electron microscopic immunohistochemistry methods, and by continuing the molecular biological characterization of these individuals.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02022-05 MDB

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular Lipid and Lipoprotein Biochemistry

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Jeffrey M. Hoeg, M.D.	Senior Investigator	MDB, NHLBI
Others:	H. Bryan, Brewer, Jr., M.D.	Chief	MDB, NHLBI
	Juan C. Monge, M.D.	Medical Staff Fellow	MDB, NHLBI
	Stephen Demosky, Jr.	Chemist	MDB, NHLBI
	Santi Datta	Chemist	MDB, NHLBI
	Briston Williamson	Lab. Tech.	MDB, NHLBI

COOPERATING UNITS *(if any)*

Drs. N.N. Tandon and G.A. Jamieson, Red Cross Research Laboratories, Bethesda, MD

LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.2	2.2	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The principal focus of this laboratory is to characterize and quantitate lipoprotein-apolipoprotein metabolism at the cellular and subcellular levels. The evaluation of cellular receptors in cultured human skin fibroblasts and hepatocytes as well as the characterization of lipoprotein receptor function in these tissues isolated from patients with inborn errors of lipoprotein and apolipoproteins metabolism provides an understanding of normal as well as deranged lipid transport in man. Development of new techniques permit the direct study of cellular process which may be coordinately controlled: cholesterol synthesis through HMG-CoA reductase, cholesteryl ester hydrolysis by both acid and neutral cholesteryl ester hydrolase, the number and affinity of lipoprotein receptors for low density lipoproteins, apolipoprotein E, and high density lipoproteins, and the synthesis of nascent apolipoproteins and lipoprotein particles. Evaluation of cellular cholesterol metabolism in homozygous familial hypercholesterolemia, Wolman Disease, cholesteryl ester storage disease, abetalipoproteinemia and Tangier disease has been undertaken in both cultured skin fibroblasts as well as with isolated hepatic tissue. We have determined that the human hepatocyte manifests at least three receptors for lipoproteins: the LDL receptor, the HDL receptor, and the apolipoprotein E receptor. Abetalipoproteinemia, homozygous familial hypercholesterolemia, Wolman disease and cholesteryl ester storage disease interrupt cellular cholesterol homeostasis at entirely different levels within the hepatocyte. The compensatory alterations which subsequently occur indicate that the pathways of de novo cholesterol biosynthesis, cholesteryl ester storage, and cholesterol incorporation into nascent lipoproteins are coordinately regulated. An understanding of human cellular cholesterol homeostasis complements the clinical studies aimed at normalizing the plasma total and low density lipoprotein cholesterol concentrations.

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Project Description:

Objective:

1) To evaluate the interaction and metabolism of lipids and lipoproteins in liver from normal and dyslipidemic humans.

The liver plays a central role in mammalian lipid and lipoprotein metabolism. The liver is not only the site of cholesterol synthesis, it is also the primary cholesterol excretion site. Furthermore, the liver synthesizes and secretes apolipoproteins and intact lipoproteins and it also removes specific lipoproteins from plasma. Although study of hepatic lipid and lipoprotein metabolism in non-human mammalian species has led to insights into physiologic and biochemical mechanisms, the obvious interspecies variation in hepatic lipid and lipoprotein metabolism has limited an understanding of the role of the human liver in normal and aberrant lipoprotein metabolism. The first step in cellular-lipoprotein interaction is the binding of the lipoprotein to specific membrane receptors. As we reported last year, normal human liver specifically binds low density lipoproteins (LDL) while individuals lacking the LDL receptors in fibroblasts (familial hypercholesterolemia homozygotes) have altered hepatic LDL recognition. By extending these hepatic lipoprotein receptor studies to include the study of other lipoproteins, to study the lipoprotein binding in other dyslipidemic conditions, and by studying human hepatocyte lipoprotein receptor regulation, we have expanded our understanding of the role of lipoprotein receptors in human liver.

Methods Employed:

1) Using the hepatic membrane isolation techniques developed last year, hepatic membranes were prepared from hepatic tissue samples taken from normolipidemic subjects and subjects with homozygotes familial hypercholesterolemia after portacaval anastomosis and abetalipoproteinemia.

2) Lipoproteins isolated by preparative ultracentrifugation and Geon-Pevikon Block electrophoresis were iodinated by the iodine monchloride method.

3) The assessment of the binding of iodinated apolipoproteins and lipoproteins to the hepatic membrane preparations was performed utilizing a Beckman Airfuge. Hepatic apolipoprotein synthesis using ^{35}S-methionine containing media in hepatic organ culture was assessed utilizing autoradiography and immunoprecipitation.

4) Methods for culturing human hepatocytes in vitro were developed. Hepatocyte lipoprotein metabolism was studied in normal and homozygous familial hypercholesterolemia homozygotes adapting fibroblast lipoprotein receptor assay techniques.

Major Findings:

1) The apolipoprotein recognition of human liver is not the same as that observed by in vitro fibroblast assay techniques. Not only are protease sensitivity, calcium sensitivity, and K_d dissimilar, but also the total loss of the fibroblast LDL receptor in homozygous FH is paralleled by only a 50% decline in LDL recognition by FH liver. Therefore, in vitro human liver has a distinctly different lipoprotein receptor system from that observed in peripheral cells.

2) The residual LDL binding observed in liver from FH homozygotes can be regulated. By performing a portacaval anastomosis, the hepatic recognition of LDL increased markedly and these changes paralleled alterations observed in the plasma LDL concentration as well as in the hepatic cholesterol and cholesteryl ester content.

3) In abetalipoproteinemia, an inborn error of metabolism in which no VLDL or LDL are present in the circulation, the hepatic recognition of LDL is markedly enhanced. This suggests that the hepatic LDL receptor(s) can be upregulated by both portacaval anastomosis as well as by depleting the pool of circulating apolipoprotein B.

4) By comparing the recognition of lipoproteins containing apoB, apoA-I, and apoE by hepatic membranes from normolipidemic subjects, FH homozygotes pre- and post-portacaval shunt, and in abetalipoproteinemia, an entire system of apolipoprotein recognition sites has emerged. ApoB binding is genetically distinct from apoE and apoA-I recognition. ApoE binding is enhanced and apoA-I binding depressed after portacaval shunt in FH. These observations indicate that human apolipoprotein recognition is a complex system of multiple lipoprotein recognition systems under separate physiologic and genetic control.

5) Modulation of human hepatocyte cholesterol content leads to alteration of both LDL and HDL receptors. The receptor number is coordinately regulated with HMG-CoA reductase activity. The in vitro hepatic membrane studies in normal and FH homozygotes were paralleled by studies performed in cultured hepatocytes from normal and FH patients. Similar to the attenuated LDL binding observed in the FH hepatic membranes, FH hepatocytes have diminished but not absent ability to bind, internalize, and degrade LDL. This nonsaturable, unregulated binding site parallels the metabolism of chemically modified LDL by normal human hepatocytes. Therefore, human liver manifests both a regulated, high affinity LDL receptor as well as a nonsaturable, unregulated LDL uptake mechanism which is independent of bulk phase pinocytosis.

6) It has been postulated that lipoproteins synthesized by the intestine during feeding are directed to the liver by a specific form of

apoB, apoB-48, whereas the liver secretes lipoproteins with a different isoform of apoB-100, which is taken up by peripheral cells. Using human hepatic organ culture incubated in media containing ^{35}S-methionine, we determined that human liver indeed secretes apoB-100. However, apoB-48 was not produced. These findings are consistent with the separate biosynthesis of B-48 and B-100 by the intestine and liver, respectively.

7) In vivo metabolic studies indicated that apoA-$I_{Tangier}$ was cleared from the plasma more rapidly than normal apoA-I. Cellular metabolism of apoA-$I_{Tangier}$ was evaluated utilizing human monocyte-macrophages, HepG-2 cells, and human skin fibroblasts. All three systems demonstrated an increased binding and degradation of apoA-$I_{Tangier}$ compared to normal. These findings suggest for the depressed levels of HDL in Tangier disease are due to enhanced degradation of lipoproteins containing apoA-$I_{Tangier}$.

Objective:

2) To study the lipoproteins and lipoprotein receptors in an animal model for homozygous familial hypercholesterolemia, the Watanabe heritable hyperlipidemic (WHHL) rabbit.

By breeding a mutant rabbit with spontaneous hyperlipidemia and accelerated atherosclerosis, Dr. Y. Watanabe of Japan developed an animal model (WHHL) for spontaneous atherosclerosis. Preliminary studies by other workers indicated that the WHHL rabbits were good models for homozygous familial hypercholesterolemia. By evaluating clinical features, adrenal function, hepatic and fibroblast receptor studies, and characterization of the plasma apolipoproteins, the role of specific apolipoproteins or the clinical features observed in this animal model could be discerned.

Methods Employed:

In vitro fibroblast and hepatic membrane LDL receptor studies utilizing ^{125}I-LDL were conducted utilizing standard techniques. Adrenal reserve was assessed by measurement of serum corticosterone before and after cosyntropin injection. Plasma lipids were quantified by standard techniques, and plasma apolipoproteins were evaluated by two-dimensional gel electrophoresis.

Adrenal and corneal histopathology and lipid content were assessed using standard techniques.

Major Findings:

1) Although the WHHL rabbit does spontaneously develop profound hyperlipidemia and accelerated atherosclerosis, the hepatic membrane and fibroblast studies do not indicate a total loss of high affinity LDL receptors. Instead, a receptor which is defective rather than absent appears to be present in this animal model.

2) The lipid and apolipoprotein evaluation disclosed patterns consistent with retention of chylomicron remnant particles in the circulation.

3) Steroidogenic tissues express LDL receptors in order to obtain LDL-derived cholesterol necessary for hormone synthesis. In this receptor-defective mutant, steroidogenesis could be impaired due to the inability of the tissue to elaborate sufficient LDL receptors. Although fasting baseline cortisol and corticosterone concentration in WHHL rabbits were the same as in normal rabbits, an attenuated response to ACTH stimulation was observed. These biochemical studies were paralleled by morphologic differences in adrenal tissue observed by light and electronmicroscopic studies. Therefore, under pharmacologic stimulation, this LDL receptor-defective mutant has impaired steroidogenic potential.

4) There has been no animal model in which to study the finding of arcus corneae which is observed in hyperlipidemic man. Using sensitive histopathologic as well as biochemical assays, cholesterol, cholesteryl ester, and triglyceride deposition at the corneal limbus was discerned in WHHL but not normolipidemic rabbits. Thus, a model for arcus corneae as well as a potential model for studying lipid and lipoprotein flux and egress has been defined.

Objective:

3) Evaluation of inborn errors in cholesteryl ester metabolism.

By evaluating tissues derived from individuals lacking a specific enzyme, the normal role of the enzyme as well as its relationship to other cellular processes can be deduced. A deficiency in acid ester hydrolase, a lysosomal enzyme which hydrolyzes cholesteryl ester and triacylglycerol bonds, can lead to Wolman Disease and Cholesteryl Ester Storage Disease (CESD). Correlation of these *in vitro* studies with the different clinical presentations of these two diseases as well as a characterization of their circulating lipids and lipoproteins could provide an understanding of the role of lysosomal cholesteryl ester metabolism on lipoprotein physiology.

Methods Employed:

Assays for acid and neutral cholesteryl ester hydrolase were developed and initially published last year. Cellular cholesterol, cholesteryl ester, and triacylglycerol content were determined using the sensitive enzymic, fluorimetric assay previously reported. Plasma lipids, lipoproteins, and apolipoprotein concentrations were quantitated using ultracentrifugation, colorimetric assays, and specific immunoassays.

Major Findings:

1) As reported last year, despite the loss of the acid esterase in Wolman Disease, the neutral esterase activity remained intact. We have subsequently found that in CESD the neutral esterase activity is increased. Therefore, neutral esterase is not only genetically distinct from acid esterase but the enhanced activity in CESD may play a role in the more mild clinical course experienced by these patients.

2) Acid esterase deficiency has a broad clinical presentation which does not appear to result from different underlying molecular mechanisms. By performing co-culture and cell fusion experiments utilizing fibroblasts derived from patients with CESD and Wolman Disease, no cross-correction of enzymic activity was found. Therefore, unlike a variety of other lysosomal storage diseases, the clinical spectrum observed in acid esterase deficiency cannot be ascribed to series of defects in enzyme metabolism which can be detected by co-culture or cell fusion techniques.

3) Marked changes in the plasma lipoproteins are observed in Acid Esterase Deficiency. These patients manifest type II hyperlipoproteinemia with increased concentrations of total cholesterol and LDL cholesterol which are in the upper 10th percentile for age and sex. This is paralleled by an increased apoB concentration and markedly decreased HDL concentrations. These alterations in plasma lipids and lipoproteins highlight the importance of lysosomal cholesterol delivery to the cell for normal lipoprotein metabolism.

4) The marked hepatomegaly and lipid accumulation observed in both CESD and Wolman Disease can now be sequentially evaluated noninvasively. The reduced radiodensity observed in the liver of these patients reflects the degree of lipid accumulation in that organ. Therefore, a noninvasive tool to assess the impact of future therapy on hepatic lipid has been established.

5) The adrenal reserve in Wolman Disease is reduced because steroidogenic tissues utilizing LDL cholesterol for the sterol backbone in hormone synthesis, a defect in the ability of LDL cholesteryl esters to undergo hydrolysis, could result in impaired steroid synthesis. Although basal and fasting serum cortisol concentrations were normal, a 36-hour ACTH infusion disclosed an attenuated rise in serum cortisol concentrations. These findings not only illustrate the importance of lysosomally derived cholesterol for normal adrenal function, they also indicate the during prolonged stress these patients may require steroid therapy.

6) The diagnosis of acid cholesteryl ester hydrolase deficiency in both Wolman Disease and CESD phenotypes can be made by evaluation of urine. Urinary renal tubular epithelial cells are shed in the urine of these subjects. Both compositional analysis and enzymic activity reliably provide the diagnosis in these diseases.

7) Repletion of Wolman and CESD fibroblasts with the enzyme acid lipase is possible. Since acid lipase is delivered to lysosomes by the mannose-6-phosphate receptor pathway, these studies suggest that enzyme replacement therapy or bone marrow transplantation could ameliorate the lipid accumulation in CESD and Wolman disease.

Objective:

4) Treatment of patients with inborn errors of lipid and lipoprotein metabolism.

Methods Employed:

Standard plasma lipid and lipoprotein quantitation techniques were used. These included enzymatic, colorimetric assays combined with ultracentrifugation. Studies in the outpatient clinic included double-blind, randomized, placebo-controlled crossover clinical trials utilizing neomycin, neomycin and niacin, neomycin and cholestyramine, and mevinolin. Radiolabeled lipoprotein turnover studies on FH homozygote patients both before and after portacaval shunt were performed to evaluate the modulation of specific apolipoprotein receptor sites in vivo. Finally, bone marrow transplantation was attempted in Wolman Disease.

Major Findings:

1) Neomycin (2g/d) reduces total and LDL cholestrol levels by 20% and 24% respectively in patients with type II hyperlipoproteinemia. The HDL cholesterol concentrations, however, were unaffected.

2) Neomycin, used as monotherapy in the treatment of type II hyperlipoproteinemia, is as affective as the conventional, FDA approved regimens for this condition (cholestyramine, colestipol, niacin).

3) No ototoxicity, nephrotoxicity or other serious side effects with long term (>3 years) treatment have been detected.

4) Combination of neomycin (2g/d) with niacin (3g/d) normalized the plasma lipoprotein concentrations in patients with type II hyperlipoproteinemia: Total and LDL cholesterol levels were markedly reduced and HDL cholesterol levels were increased.

5) In contrast, combination of the bile acid sequestrant cholestyramine with neomycin treatment did not have the anticipated additive effects on the lipoprotein cholesterol levels.

6) Mevinolin, a competitive inhibitor of the enzyme catalyzing the rate-limiting step in cholesterol biosynthesis HMG-CoA reductase, is the single most effective agent to normalize plasma lipoprotein concentrations which we have evaluated.

7) Although mevinolin could theoretically impair adrenal steroidogenesis, 20 mg bid of mevinolin did not significantly alter adrenal reserve in 20 type II hyperlipoproteinemia patients.

Significance to Biomedical Research and the Program of the Institute:

By evaluating the interaction of lipoproteins with intact human cell lines and isolated subcellular fractions from different tissues in vitro, insights on the normal physiological lipid transport function of the lipoproteins and their coordination with intracellular lipid metabolism can be derived. In addition, these studies can be extended to evaluate possible pathophysiologic mechanisms of the dyslipoproteinemias. Information on specific molecular defects in the dyslipidemias and the metabolic consequences of these defects is necessary for an understanding of these disease processes and may ultimately lead to more effective treatment for these disorders. In addition, these findings provide insight that could be more generalized to the understanding and prevention of atherosclerosis and coronary artery disease.

Proposed Course:

Utilizing the information we have generated outlining the coordinate control of cellular cholesterol content, the activity of intracellular enzymes associated with cellular cholesterol metabolism, and the lipoprotein receptors characterized in human liver, we will assess the role of these pathways on nascent lipoprotein particle synthesis in human liver. Immunoblot techniques, radiolabeled pulse-chase experiments and newly developed molecular probes for all of the critical human apolipoproteins will all be focused upon questions related to the coordinate regulation of human hepatic apolipoprotein and lipoprotein synthesis. Ongoing evaluations of strategies to reduce atherogenic lipoprotein particle concentrations in type II hyperlipoproteinemia will continue.

Publications:

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22. Wilson, W.F., Zech, L.A., Gregg, R.E., Schaefer, E.J., Hoeg, J.M., Sprecher, D.L., Brewer, H.B. Jr.: Estimation of VLDL cholesterol in hyperlipidemia. Clin Chim Acta (in press), 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02024-04 MDB

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of Plasma Apolipoproteins and Lipoproteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Neme, title, laboratory, and institute affiliation)*

PI:	Simon W. Law	Senior Investigator	MDB, NHLBI
Others:	H. Bryan Brewer, Jr. M.D.	Chief	MDB, NHLBI
	Silvia Fojo, M.D.	Clinical Associate	MDB, NHLBI
	Karl Lackner, M.D.	Visiting Scientist	MDB, NHLBI
	Richard Gregg, M.D.	Senior Investigator	MDB, NHLBI
	Jeffrey Hoeg, M.D.	Senior Investigator	MDB, NHLBI
	Susanne Czarnecki, Ph.D.	Post-Doctoral Fellow	MDB, NHLBI

COOPERATING UNITS *(if any)*

A. Sakaguchi & S. Naylor - Departments of Medicine and Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas

LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.3	5.3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The entire amino acid sequence of Tangier preproapoA-I has been determined. The sequence of Tangier preproapoA-I was identical to normal preproapoA-I except for a single base substitution (G → T) which resulted in the isosteric replacement of a glutamic acid residue at position 120 to aspartic acid. Results indicate the rapid rate of catabolism of apoA-I in Tangier disease is due to a post-translational defect in apoA-I metabolism rather than a structural defective apoA-I.

Human liver apoB-100 cDNA has been isolated in a λgt-11 expression library. The apoB-100 mRNA is 15-18 kb enough to encode a protein of M.W. 400 kd. Computer analysis of nucleic acid derived amino acid sequence of cloned apoB-100 cDNA predict a significant portion of the protein is organized into βstructure which may be important in lipid apoB-100 interactions in LDL and contributed to the insolubility of delipidated apoB-100 in aqueous buffers. In collaboration with S. Naylor and associates, we have localized the apoB-100 gene to the p23→pter region of chromosome 2. The cloning of human apoB-100, has provided new insights into the structural and physicochemical properties of apoB-100, and enable studies to be initiated on the factors modulating apoB-100 biosynthesis and the apoB-100 gene in patients with dyslipoproteinemias.

Two additional apolipoproteins, C-III and E have been cloned. ApoE gene expression in culture monocyte-macrophages derived from normal subject and apoE deficient patient was evaluated. Cellular apoE mRNA level was greatly reduced. Furthermore no major deletion or insertions were detectable in the apoE gene in the E deficient patient. Thus the deficiency of plasma apoE in the patient with apoE deficiency is due to a markedly decreased level of apoE mRNA and decreased synthesis of the E apolipoprotein.

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Project Description:

Objectives:

1). Evaluation of apoA-I gene in Tangier patient.

Methods Employed:

Messenger RNA (mRNA) was isolated from liver biopsy using the guanidine thiocyanate/Cscl cushion centrifugation procedure. A liver cDNA library was establish in E. coli using plasmid pBR322 as cloning vector. ApoA-I Tangier cDNA clones was identified by colony filter hybridization with a normal apoA-I cDNA probe. DNA sequences were determined by the Maxam-Gilbert procedure. Synthetic oligodeoxynucleotides were used in a primer-extension procedure to determine the remaining sequence at the 5' portion of Tangier apoA-I mRNA molecule.

Major Findings:

ApoA-I mRNA level in a Tangier patient's liver tissue was found similar to normal. The entire amino acid sequence of Tangier preproapoA-I has been derived by nucleic acids sequencing of cDNA clones isolated from Tangier liver cDNA library and by primer-extensions on Tangier liver RNA. The sequence of Tangier preproapoA-I was identical to normal preproapoA-I except for a single base substitution (G → T) which resulted in the isosteric replacement of a glutamic acid residue at position 120 to aspartic acid. Our findings are interpreted as indicating that there is no major structural defect in Tangier apoA-I and the rapid rate of catabolism of apoA-I in Tangier disease is due to a post-translational defect in apoA-I metabolism.

Objectives:

2). Molecular cloning of human apoB-100 mRNA.

Methods Employed:

ApoB-100 cDNA clones were identified from a human liver λgt-11 cDNA expression library utilizing monospecific apoB-100 antibody, monoclonal antibody as well as synthetic oligodeoxynucleotides as probes. Lysogens were established in E. coli. Y1089 and apoB-100 polypeptides were isolated from IPTG induced cell cultures as a hybrid protein with β-galactosidase. Southern, Western and Northern blot filter hybridization were performed by standard procedure.

Major Findings:

Human apoB-100 cDNA clones have been identified in a human liver λgt-11 cDNA expression library. ApoB-100 specific cDNA probes were isolated from several cDNA clones. Northern blot filter hybridization of liver RNA revealed the apoB-100 mRNA is 15-18 kb long which is of sufficient size to code for an apolipoprotein of 250,000 to 387,000 daltons, the proposed

molecular weight of delipidated plasma apoB-100. The complete nucleotide and derived amino acid sequence of a 1.6 kb cDNA predict a significant portion of the protein is organized into structure which may be important in lipid apoB-100 interactions in LDL and contributed to the insolubility of delipidated apoB-100 in aqueous buffers. In collaboration with Drs. A. Skaguchi and S. Naylor, we have localized the apoB-100 gene to the p23 → pter region of chromosome 2. Evaluation of Southern and Northern blot filter hybridization indicate no major deletions or insertions in the apoB-100 gene of a patient with abetalipoproteinemia and that mRNA of similar size was expressed in liver tissue obtained from this patient. Detail structural analysis of apoB-100 genes are currently underway.

Objective:

3). Evaluate the apoE gene in apoE deficient patient.

Methods Employed:

Synthetic oligonucleotides were employed as hybridization probes to screen our human liver cDNA library. A 1.1 kb apoE cDNA insert was isolated from clone pMDB760 and used in Northern blot filter hybridization analysis of RNA isolated from cultured monocyte-macrophages of normal and apoE deficient patient. The apoE gene in normal and in a apoE deficient patient were analysed by the method of Southern.

Major Findings:

The apoE gene is present in the apoE deficient patient, and there are no major insertions or deletions in the gene by Southern blot analysis. Monocyte-macrophages isolated from a patient with apoE deficiency contain 1-3% of the level of apoE mRNA present in monocyte-macrophages isolated from normal subjects. The small quantity of apoE mRNA in the apoE deficient monocyte-macrophages is similar in size to normal apoE mRNA. The deficiency of plasma apoE in the patient with apoE deficiency is due to a markedly decreased level of apoE mRNA and decreased synthesis of the E apolipoprotein. The decreased apoE mRNA may be due to a defect in transcription of the apoE gene or to instability of the apoE mRNA. Further analysis is currently underway.

Objectives:

4). Isolation of apolipoprotein C-III cDNA.

Methods Employed:

ApoC-III cDNA clones have been identified by hybridization with synthetic oligonucleotides as previously described. DNA sequence was determined by the Maxam and Gilbert procedure.

Major Findings:

ApoC-III cDNA clones have been identified and sequenced. The availability of apoC-III cDNA probe is important to our undersanding the coordinate regulation of the expression of the linked A-I and C-III gene complex.

Significance to Biomedical Research and the Program of the Institute:

The cloning and nucleic acid sequence analysis of human apolipoproteins provided new insights to the biosynthesis and processing of the plasma apolipoproteins. Using cloned cDNA as hybridization probes enabled us to initiate studies on gene organization and expression in patients with dyslipoproteinemias such as Tangier disease and apoE deficiencies. Our findings enable us to better understand the genetic defect of these two dyslipoproteinemias and help to direct future research activities on these and related diseases. The identification of apoB-100 cDNA clones enabled us to a) Initiate detailed analyses of the apolipoprotein B gene and protein structure. b) To elucidate the biosynthesis and processing of apoB-100. c) The coordinate control of the expression of apoB-100 and the LDL receptor and d) The characterization of the apoB-100 gene in abetalipoproteinemia and other dyslipoproteinemias.

Proposed Course:

Studies will be continued on the analysis of apoE gene and its expression in apoE deficient patients. Detail molecular analysis such as sequence comparison with the normal apoE gene, methylation patterns, DNase 1 and other nucleases sensitivities and maturation of mRNA will be initiated. Rapid deletion subcloning (RDS) system for dideoxy sequencing will be used in parallel with the Maxam-Gilbert chemical modification procedure to establish the entire mRNA sequence of apoB-100. Computer analysis and modeling will be used to help better understand apoB-100 protein structure and function. ApoB-100 cDNA clones will be used as probes to study gene expression and regulation of apoB-100 and the LDL receptor in vitro using dot hybridization techniques. The characterization of apoB-100 gene in abetalipoproteinemia and other dyslipoproteinemias is currently underway.

Publications

1. Law, S.W., and Brewer, H.B., Jr.: Nucleotide sequence and the encoded amino acid of human apolipoprotein A-1 mRNA. Proc. Natl. Acad. Sci. USA. 81:66-70, 1984.

2. Lackner, K.J., Law, S.W., and Brewer, H.B. Jr.: Human apolipoprotein A-II: Complete nucleic acid sequence of preproapoA-II. FEBS Lett. 175:159-164, 1984.

3. Fojo, S.S., Law, S.W., and Brewer, H.B., Jr.: Human apolipoprotein C-II: Complete nucleic acid sequence of preapolipoprotein C-II. Proc. Natl. Acad. Sci. USA. 81:6354-6357, 1984.

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7. Fojo, S.S., Law, S.W., Brewer, H.B. Jr., Sakaguchi, A.Y., and Naylor, S.L.: The localization of the gene for apolipoprotein C-II to chromosome 19. Biochem. Biophys. Res. Commun. 122:687-693, 1984.

8. Lackner, K.J., Law, S.W., and Brewer, H.B., Jr.: The human apolipoprotein A-II gene: complete nucleic acid sequence and genomic organization. Nucl. Acids. Res. 13:4597-4608, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02027-01 MDB

PERIOD COVERED: October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*: Molecular Biology of the ApoA-II Gene

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Karl J. Lackner, M.D.	Visiting Fellow	MDB, NHLBI
Others:	Simon W. Law, Ph.D.	Senior Investigator	MDB, NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI

COOPERATING UNITS *(if any)*

A. Sakaguchi & S. Naylor - Departments of Medicine and Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas

LAB/BRANCH: Molecular Disease Branch

SECTION: Peptide Chemistry

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The entire nucleic acid sequence of the human apoA-II gene has been determined. The structure of the apoA-II gene is similar to that of other apolipoproteins, like apoA-I, apoE, and apoC-III. The apoA-II gene consists as the aforementioned genes of three introns and four exons. The position of the introns is similar in all four genes. Promoter regions as well as a possible Z-DNA element have been identified. The knowledge of the structure of the gene will allow to study the expression in more detail.

To better understand the processing of apoA-II its isoforms have been analyzed in human thoracic duct lymph and plasma as well as in culture media from normal hepatocytes and the hepatoma cell line HepG2. ProapoA-II and several isoforms of apoA-II including sialylated forms were identified. ProapoA-II has a strikingly basic pI of 6.79 which is caused by three additional positively charged residues as compared to mature apoA-II. The major mature isoform has a pI of 4.90. The sialoforms are more acidic and have a slightly higher apparent molecular weight. The isoform pattern in several dyslipidemias is different from the normal control. The reason for this variability is not yet fully understood.

The apoB-100 gene and mRNA have been analyzed in normal subjects and abetalipoproteinemic (ABL) patients. The apoB gene is present in ABL and not structurally different from normal controls as determined by Southern blot analysis. The apoB-100 mRNA in ABL is of the same size as the normal mRNA, but its concentration is reduced. The absence of apoB from the plasma of ABL-patients, however, cannot be explained by this reduction. We conclude that the defect in ABL is post-translational and additional studies to characterize the defect are being undertaken.

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Project Description:

Objectives:

1) Structural analysis of the apoA-II gene

Methods Employed:

The apoA-II gene was isolated from a human genomic library cloned in Charon 28. The library was screened with a previously isolated apoA-II cDNA probe to detect specific clones, using a filter hybridization procedure. Positive clones were analyzed by Southern blotting and grown in large scale. A restriction fragment containing the complete apoA-II gene was subcloned into plasmid pBR322 and sequenced by the chemical cleavage method of Maxam and Gilbert.

Major Findings:

The gene for human apoA-II was isolated from a human genomic library. A clone containing the gene and 9.0 kb upstream and 3.5 kb downstream was identified. The complete nucleic acid sequence of the apoA-II gene has been determined. The apoA-II gene is interrupted by three intervening sequences of 169,293 and 395 bp respectively. The second intron is of particular interest, because it contains a 33 bp long sequence of GT residues next to its 3' splice site. Alternating purime-pyrimidine sequences have been shown to form Z-helix structure in vivo. A restriction fragment length polymorphism 3' from the apoA-II gene has been identified, which may serve as a marker in studies of genetic linkage.

Objectives:

2) Analysis of apoA-II isoforms in plasma and lymph.

Methods Employed:

ApoA-II isoforms were analyzed by two dimensional gel electrophoresis. The first dimension was an isoelectrofocussing pH gradient, the second dimension was NaDodSO$_4$-PAGE. To identify apoA-II, Western blots were probed with monospecific antisera against apoA-II. Since proapoA-II is the only isoform of apoA-II, that contains arginine, HepG2 cells were incubated with ^{14}C-arginine and the secreted apolipoproteins analyzed. To identify sialoforms of apoA-II, apolipoproteins were incubated with neuraminidase and compared to untreated controls by 2D-PAGE.

Major Findings:

ProapoA-II and several isoforms of apoA-II including sialoforms were identified. ProapoA-II has a pI of 6.79, which is considerably more basic than that of the major plasma isoform with pI 4.90. The other isoforms had pI values of 5.17, 4.68, 4.42, and 4.20 respectively. The sialoforms had a

slightly higher apparent molecular weight and additional negative charges equal to their number of sialic acid residues. The relative concentrations of isoforms was different in lymph, and plasma HDL, LDL, and VLDL. Also, in some dyslipoproteinemias the isoform pattern was different from normal controls.

Objectives:

3) Analysis of the apoB gene and mRNA in abetalipoproteinemia (ABL)

Methods Employed:

Genomic DNA was isolated from leukocytes or liver cells. The apoB gene was analyzed using radiolabeled cDNA fragments from apoB specific clones as probes on Souther blots. RNA was isolated from normal and ABL liver using the guanidinium isothiocyanate procedure with subsequent purification of RNA by a CsCl gradient centrifugation. Poly(A)$^+$ RNA was prepared by oligo-dT affinity chromatography. The apoB mRNA was identified by Northern blotting and quantitated by Dot blotting.

Major Findings

The presence of an apoB mRNA of normal size in ABL could be established. The measured level was reduced to approximately 50% of normal in the sample from ABL liver. Southern blot analysis of the apoB gene in ABL did not reveal any major abnormalities as compared to normal. Thus it could be shown that ABL is most likely caused by a post-translational defect in the processing of apoB, rather than a promoter defect or other defect on the transcriptional level.

Significance to Biomedical Research and the Program of the Institute:

The knowledge of the structure and organization of the apoA-II gene will enable us to analyze the regulation of expression of the gene. The analysis of the plasma isoforms provides important insight into the processing of apoA-II after secretion. Thus the results obtained of these studies will be important for the understanding of HDL synthesis, secretion and metabolism.

The analysis of the apoB-100 gene in ABL provided important new insights into the nature of the genetic defect of ABL. Thus it may become possible to define the defect, which has been poorly understood until now.

Proposed Course:

Studies to analyze the regulation of the apoA-II gene in cultured hepatoma cells and hepatocytes will be undertaken. The influence of different culture conditions as well as drugs will be analyzed.

The degree of methylation of the apoA-II gene is currently under investigation, and preliminary data suggest that methylation of the gene is

related to its inactivation. We are also planning to further define the post-translational changes apoA-II undergoes within the cell and in lymph and plasma. This will provide better insight into the metabolism of apoA-II and HDL.

Genetic linkage analysis will be performed on ABL patients to gain further data on the potential defect in this disease. Cell free translation of apoB mRNA from normal and ABL subjects is planned. This will allow the analysis of the primary translation product in ABL.

Publications

1. Lackner, K.J., Law, S.W., and Brewer, H.B. Jr., Sakaguchi, A.Y., and Naylor, S.L.: The human apolipoprotein A-II gene is located on chromosome 1. Biochem. Biophys. Res. Comm. 122:877-883, 1984.

2. Lackner, K.J., Law, S.W., and Brewer, H.B., Jr.: Human apolipoprotein A-II: Complete nucleic acid sequence of preproapoA-II. FEBS Lett. 175:159-164, 1984.

3. Lackner, K.J., Edge, S.B., Gregg, R.E., Hoeg, J.M., and Brewer, H.B., Jr.: Isoforms of apolipoprotein A-II in human plasma and thoracic duct lymph. J. Biol. Chem. 260:703-706, 1985.

4. Lackner, K.J., Law, S.W., and Brewer, H.B., Jr.: The human apolipoprotein A-II gene: Complete nucleic acid sequence and genomic organization. Nucl. Acids Res. 13:4597-4608, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02028-01 MDB

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biology of the ApoC-II Gene

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Silvia S. Fojo, Ph.D., M.D.	Medical Staff Fellow	MDB, NHLBI
Others:	Simon W. Law, Ph.D.	Senior Investigator	MDB, NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB, NHLBI

COOPERATING UNITS *(if any)*

Carlo Gabelli, M.D., and Giovanella Baggio, M.D. - University of Padova, Padua Italy

LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The complete coding sequence of preapolipoprotein C-II has been determined from an apoC-II clone isolated from a normal human liver cDNA library. PreapoC-II contains a 22 amino acid signal peptide attached to the 79 amino acid mature protein. This is similar to other secretory proteins that are synthesized as precursors and undergo subsequent modfication. In collaboration with A.Y. Sakaguchi and associates, we have localized the apoC-II gene to chromosome 19.

Analysis of the apoC-II gene in apoC-II deficiency was done by hybridization following Southern blotting of genomic DNA from 2 independent patients with apoC-II deficiency. The apoC-II gene is present in these patients with no major insertions or deletions. Analysis of the plasma in the apoC-II deficient patients by 2 dimensional gel electrophoresis and Western blotting revealed the presence of apoC-II variants with abnormal electrophoretic mobility. Family studies suggests that the proband from one of the apoC-II deficient families is a compound heterozygote.

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Project Description:

Objectives:

1) Structural analysis of the apoC-II gene.

Methods Employed:

A normal human liver cDNA library previously established in E.Coli using plasmid pBR322 as cloning vector was screened for apoC-II clones. These were identified by colony filter hybridization with a 17 base-long synthetic oligonucleotide probe based on amino acids 5-10 of apoC-II. DNA sequences were determined by the Maxam-Gilbert procedure. Chromosomal localization was determined by filter hybridization analysis of human-mouse hybrid cells.

Major Findings:

The complete nucleic aicd sequence of preapoC-II mRNA has been determined. The apoC-II clone isolated contained 500 bp that encoded for 101 amino acids of preapoC-II, which included a 22 amino acid signal peptide and a 79 amino and mature protein. The finding of an apoC-II prepeptide is consistent with the fact that apoC-II is a secretory protein. A comparison of the amino acid sequence of preapoC-II and proapoA-I reveals a sequence of 5 identical amino acids at the processing site of proapoA-I to apoA-I. This raises the possibility that preapoC-II is indeed synthesized as a preproapolipoprotein C-II. In collaboration with Drs. A. Sakaguchi and S. Naylor the apoC-II gene has been localized to chromosome 19. It is of interest that the gene for apoE and the LDL recpetor are present in this same chromosome.

Objectives:

2) Analysis of the apoC-II gene and protein in apoC-II deficiency.

Methods Employed:

Genomic DNA from 2 patients with apoC-II deficiency was isolated from white blood cells and subjected to restriction endonuclease digestion. Hybridization following Southern blotting was done by utilizing a nick-translated probe isolated from the apoC-II cDNA clone. Analysis of apoC-II in the plasma of the 2 apoC-II deficient probands was done by 2 dimensional gel electrophoresis followed by Western blotting with a rabbit anti-apoC-II antibody.

Major Findings:

ApoC-II gene is present in 2 independent patients with apoC-II deficiency. No major insertions or deletions were detected in the apoC-II gene of these patients by Southern blot analysis. Although minor changes in the nucleotide sequence cannot be excluded, major rearrangements in the

structural gene of apoC-II were ruled out. Decreased levels of an apoC-II variant with abnormal electrophoretic mobility was detected in the plasma of both patients with apoC-II deficiency by Western blotting. In one of the apoC-II deficient families, the father of the proband has a null allele, the mother has an apoC-II variant allele and the proband is a compound heterozygote having inherited the two abnormal alleles for the parents.

Significance to Biomedical Research and the Program of the Institute:

The determination of the complete cDNA sequencce of apoC-II and the availability of a cDNA probe of apoC-II will facilitate our analysis of the biosynthesis and processing as well as the genomic organization of apoC-II in normal subjects and patients with dyslipoproteinemias. Our studies of the apoC-II gene in patients with apoC-II deficiency enable us to better understand the genetic defect in this and other disorders of triglyceride metabolism. Our detection of a variant apoC-II in these patients enhance our understanding of apoC-II biosynthesis and processing.

Proposed Course:

Studies are underway to elucidate the complete genomic sequence of normal apoC-II and to establish genomic libraries from the DNA of the 2 apoC-II deficient patients. A comparison of these sequences should detect any differences in the structural or regulatory areas of the apoC-II gene in these patients. Analysis of the liver mRNA levels in the apoC-II deficient proband will be done by Northern blot filter hybridization to determine the size and levels of the apoC-II mRNA.

Publications

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3. Fojo, S.S., Law, S.W., Sprecher, D.L., Gregg, R.E., Baggio, G., and Brewer, H.B. Jr.: Analysis of the apoC-II gene in apoC-II deficient patients. Biochem. Biophys. Res. Commun. 124:308-313, 1984.

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Annual Report of the
Laboratory of Molecular Hematology
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1985

The Laboratory of Molecular Hematology (LMH) is involved in studying the basic molecular mechanisms of gene expression and protein synthesis, specifically using hemoglobin and adenosine deaminase (ADA) as a model system. LMH is composed of three segments: the Section on Molecular Genetics, which is primarily concerned with the molecular control of eukaryotic gene expression; the Section on Molecular Cloning, which is primarily concerned with the isolation and characterization of regulatory sequences that control globin and other genes from the genomes of eukaryotic cells; and the Section on RNA and Protein Biosynthesis, which is primarily concerned with the mechanism and regulation of hemoglobin synthesis at the transcriptional and translational levels. LMH is closely associated with the Clinical Hematology Branch (CHB) and collaborates on a number of joint projects.

The objectives of this laboratory are to: (1) identify, isolate and characterize the regulatory factors of animal and human tissues that are involved in the control of the expression of the genes, particularly the globin genes; (2) analyze the genomic DNA sequences involved in the regulation of gene expression in humans and animals; (3) develop methods for transferring functional genes into tissue culture cells and intact animals; (4) develop and characterize animal and tissue culture model systems for human genetic anemias and immune deficiency diseases; (5) characterize the molecular basis of translational regulation; and (6) examine the overall regulation of gene expression in normal, experimental, and disease (e.g., thalassemia and ADA^- immune deficiency) states. Information from these programs is used to study the regulation of globin and ADA gene expression in normal and a mutant mouse (and human) DNA. The long-term goal is to develop a means whereby gene defects can be corrected in mice, in monkeys, and, ultimately, in patients with β-thalassemia, severe combined immunodeficiency disease (SCID) and other diseases involving genetic defects.

SECTION ON MOLECULAR GENETICS

Retroviral techniques and recombinant DNA technology have been used to construct retroviral vectors expressing the bacterial neo^R gene and the human ADA gene. A highly efficient procedure for transferring functional genes into mammalian cells has been developed using retroviral-based vectors as a delivery system. When mouse bone marrow cells are infected in vitro with a neo^R gene and reinjected into a lethally irradiated recipient mouse, 90% of the stem cells (CFU-S) can be shown to carry an intact copy of the neo^R gene. The majority of these cells produce the neo^R gene product: phosphotransferase. Retroviral vectors containing the human gene for the enzyme adenosine deaminase (ADA) have been made and inserted into mice, monkeys, and ADA^- human T and B cells. These studies are preliminary to attempting human gene therapy in patients suffering from ADA^- severe combined immunodeficiency disease.

This Section has succeeded, during the past year, in demonstrating that:

(1) A retroviral vector containing a neo^R gene can be stably integrated into the bone marrow cells of mice at a high efficiency. The neo^R gene expresses

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efficiency in the bone marrow and blood cells of a long-term reconstituted animal.

(2) A retroviral vector containing the human ADA gene can be stably integrated into murine and primate hematopoietic cells where it expresses efficiently.

(3) Primates can be transplanted with autologous bone marrow cells treated with the ADA retroviral vector in a protocol similar to what would be carried out for human gene therapy.

(4) B and T cells from a patient suffering from ADA$^-$ SCID can be corrected by the ADA retroviral vector in vitro.

SECTION ON MOLECULAR CLONING

A multifaceted approach is being taken to study the regulation of the mouse β-globin gene: vectors have been developed to assay β-globin promoter function; these vectors have been used to determine the ability of DNA sequences to increase expression from this promoter. Vectors have been introduced into tissue culture cells to create stable transformants in order to study both the β-globin promoter and the activity of DNA enhancers on that promoter. In addition to tissue culture studies, we have introduced vectors into mice to look for stable integration of the plasmid DNA and activity of the cloned mouse β-globin promoter. Finally, in vitro analysis of protein extracts has revealed a specific protein fraction that exhibits binding activity to a DNA enhancer. The knowledge from these studies, ranging from in vitro assays to expression in tissue culture cells and finally expression in mice, are giving us a better understanding of the regulation of the β-globin gene and DNA sequences which affect the β-globin promoter.

This Section has succeeded, during the past year, in demonstrating that:

(1) DNA sequences from two viruses, RSV and SFFV, enhance expression from the mouse β-globin promoter in an expression vector we have developed.

(2) A mouse DNA sequence inserted into the β^{maj}-globin gene locus in a thalassemic mouse also has enhancing ability in our expression vector system.

(3) Expression from the mouse β-globin promoter on integrated plasmids can be increased by enhancers at low plasmid copy numbers, but at higher copy numbers expression decreases and is dependent only on the copy number.

(4) Our expression vector DNA can be successfully transferred to mice and stably transmitted through four generations.

(5) DNA enhancers have a potential secondary structure. An assay to detect enhancer mutants has been developed.

(6) A protein fraction from HeLa cells binds specifically to the SV40 enhancer.

SECTION ON RNA AND PROTEIN BIOSYNTHESIS

To understand the regulation of gene expression at the level of transcription, an active HeLa whole cell extract is being purified into individual factors required for correct initiation by RNA polymerase II. Purified factors will be

used to reconstitute in vitro systems in which the mechanisms that influence gene activity can be studied. Such information is important to achieve the successful design of vectors in gene therapy capable of regulated and appropriate expression. In addition, previous studies on regulation at the translational level have demonstrated that regulatory mechanisms first discovered in the reticulocyte lysate system are, in fact, general to all cell types and are involved in a variety of viral infections.

During the past year, this Section has achieved the following results:

(1) A presumptive 90,000 dalton transcription initiation factor has been identified as a component of Adenovirus 2 major late promoter preinitiation and initiation transcription complexes and has been purified to apparent homogeneity from HeLa whole cell transcription extracts.

(2) The phenotypic compensation in the murine β-thalassemic model has been shown to be the result of a translational rather than transcriptional mechanism.

(3) A rapid method for separating α and β globin chains by reverse phase HPLC has been developed.

(4) Using oligonucleotide probes specifying coding sequences within the first 23 NH_2-terminal amino acids of eIF-2α, positive eIF-2α clones have been identified in rabbit recombinant genomic DNA bacteriophages.

(5) Influenza and adenovirus infection overcome interferon-mediated host defenses by producing gene products which prevent activation of the double-stranded RNA dependent eIF-2α kinase.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02213-08 MH

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of RNA and Protein Biosynthesis in Cell-Free Systems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	B. Safer	Medical Officer	LMH, NHLBI
Others:	W. F. Anderson	Chief	LMH, NHLBI
	H.E. Tolunay	Senior Staff Fellow	LMH, NHLBI
	T. Brendler	Staff Fellow	LMH, NHLBI
	M. Schafer	Chemist	LMH, NHLBI
	W. Kemper	Chemist	LMH, NHLBI
	L. Yang	Biologist	LMH, NHLBI
	M. Joan Curcio	Biol. Lab. Tech.	LMH, NHLBI
	S. Garfinkel	Biol. Lab. Tech.	LMH, NHLBI
	T. Boal	Guest Worker	LMH, NHLBI
	J. A. Thompson	Expert	LMH, NHLBI

COOPERATING UNITS *(if any)*
Robert Krug, Memorial Sloan-Kettering Cancer Center, New York, NY; Tom Shenk, Princeton University, Princeton, NJ.

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section on RNA and Protein Biosynthesis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.5	2.6	3.9

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Regulation of gene expression occurs at the level of transcription, processing, transport and mRNA translation. The primary goals of this section are to investigate the transcriptional and translational control mechanisms responsible for regulated gene expression.

To identify components required for transcription of mRNA by RNA polymerase II, distinct preinitiation, initiation and elongation transcription complexes have been purified and characterized. The protein factors associated with the specific DNA fragment containing promoter and control sequences are being used to 1) identify transcription factors and 2) to generate monoclonal antibodies to identify their partial functional activities during transcriptional initiation. Factors are being purified by DNA affinity chromatographic techniques, under conditions where we have demonstrated specific interactions between protein and DNA promoter sequences required for transcriptional initiation. A 90 kD polypeptide complex has been purified to apparent homogeneity using these techniques and is currently being evaluated for its function during transcription.

The simplicity of globin gene expression in a mouse model of β-thalassemia has been useful in examining the regulation of translation and transcription in vivo. Deletion of the β-major globin gene is accompanied by a compensatory increase in β-minor globin gene expression. The ratio of β-minor:α globin, normally 0.2, increases from 0.2 to 0.8. However, compensation is almost totally the result of increased translational initiation on β-minor globin mRNA since the ratio of β-minor to α transcriptional initiation and mRNA remain at 0.2.

To understand the regulation of translation factors, the genes for the α, β, and γ subunits of eIF-2 are being identified and sequenced. Oligonucleotide probes based on the eIF-2α amino-terminal sequences have tentatively identified genomic clones for eIF-2α. In addition, the mechanism of translational regulation during adenovirus and influenza viral infection has been shown to involve the modulation of eIF-2 dsRNA kinase activity.

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Project Description:

Objectives: The major goals are: (1) to determine the sites and mechanisms of transcriptional and translational control of gene expression; (2) to identify and characterize the components involved in such regulation; (3) to develop active *in vitro* translation and transcription systems which retain key regulatory features found in intact cells; (4) to clone and sequence genes for eukaryotic translational initiation factors, initially eIF-2; and (5) to identify the role of host translation initiation factor modification during viral infection.

Methods Employed: Conventional chromatographic procedures, HPLC reverse phase and affinity chromatography using antibodies prepared against purified initiation factors are used to rapidly isolate key translational and transcriptional components under defined experimental conditions. Covalent modification of these regulatory factors will be examined by incorporation of specific radioisotopes, direct chemical analogs, group specific reagents, and by alteration of their physical characteristics (e.g., pI, S_{20w}). Active translation and transcription systems have been developed for K-562 cells using non-disruptive procedures which maintain cell ultrastructure. Large scale culture of K-562 cells will be used to obtain the large amounts of cells required for purification of translational and transcriptional components. Transcription factors recognizing specific promoter sequences are identified by isolation of discrete DNA protein complexes on low ionic strength polyacrylamide gels or binding of specific radiolabeled DNA fragments to nitrocellulose filters. Binding sites are being mapped by DNase I and dimethylsulfate footprinting. Proteins are subjected to automated Edman degradation to generate N-terminal amino acid sequence information. These partial sequences are used to synthesize mixed oligonucleotide probes using automated solid phase phosphotriester chemistry. The probes are purified via HPLC reverse phase chromatography. Urea-PAGE gels are used to check purity and probe size. The probes are tested for hybridization via Southern and dot blot analysis. The eIF-2 probes are then used to screen genomic and cDNA libraries. The selected clones will be evaluated via restriction enzyme mapping, hybridization-selected mRNA translation and partial sequence analysis. Once the desired genes have been isolated, sequence analysis and electron microscopic R-loop analysis can be utilized to elucidate the genetic organizational structure and the sequences responsible for the primary structure of the eIF-2 protein. mRNA is measured by hybridization to specific α and β globin probes generated using Sp6 and M13 vectors. Hybrids are analyzed by dot blot and nuclease protection mapping. Functional mRNA translation intermediates are fractionated by sucrose density gradient centrifugation. Separation of α and β globin chains translated *in vivo* and *in vitro* are rapidly separated by HPLC reverse phase chromatography.

Major Findings:

1. A presumptive transcription initiation factor has been purified to apparent homogeneity from HeLa whole cell transcription extracts using the differential nitrocellulose filter binding assay. The native M_r = 90,000 and the protein consist of 2 polypeptide subunits of 54 and 36 kD. Footprinting studies and *in vitro* transcription assays are currently underway to identify its function during transcription initiation.

Major Findings (continued):

2. The 54 and 36 kD subunits described above have been identified as polypeptide components of Adenovirus 2 major late promoter preinitiation and initiation transcription complexes isolated by gel-filtration chromatography.

3. Other transcriptionally active protein fractions of HeLa and MEL cell extracts which form specific DNA/protein complexes have been identified. These are currently being purified by FPLC chromatography for further evaluation as transcription initiation factors.

4. A multipurpose chromatographic support matrix for the rapid ligation of cloned DNA fragments has been developed for purification of DNA binding proteins by affinity chromatography.

5. The phenotypic compensation in the murine β-thalassemic model is the result of a translational rather than transcriptional mechanism.

6. Translation is initiated more rapidly on β minor mRNA than α globin mRNA. RNase T1 analysis of reticulocyte polysomes resolved by sucrose gradient centrifugation shows that, although the ratio of β minor to α in total reticulocyte mRNA remains at a value of 0.2-0.3 found in the normal mouse, the β minor to α ratio of polysomal mRNA is 4 times higher (0.8). Possible alterations of initiation factor activities in the thalassemic reticulocytes are being studied.

7. A rapid method for separating α and β globin chains by reverse phase HPLC has been developed.

8. Oligonucleotide probes specifying coding sequences within the first 23 NH_2-terminal amino acids of eIF-2α have been rapidly synthesized and purified using reverse phase HPLC methodologies.

9. Positive eIF-2α clones have been identified in rabbit recombinant genomic DNA bacteriophages.

10. The usefulness of our capability to synthesize DNA fragments was expanded by collaborations with various other scientists. Both mixed oligomers (composed of numerous short DNA chains) and single stranded DNA fragments were synthesized for scientists in other laboratories at the NIH (Joel Moss, Michael Zasloff, John Folk) as well as for various scientists in our own laboratory.

11. An additional collaboration was undertaken involving the elucidation of the structural organization of a Type 1 mouse Keratin gene via electron microscopic R-loop analysis. The fragmented gene was found to contain 8 exons, the size range was from 120 to 655 bp, separated by 7 introns whose sizes ranged from 138 bp to 611 bp. By R-loop analysis, this genomic clone was shown to encode the complete sequence of the gene which was approximately 4.5 kb in length.

12. Influenza virus and adenovirus overcome interferon-mediated host antiviral activity by producing a gene product that prevents activation of the double-stranded RNA dependent eIF-2 kinase.

Significance to Biomedical Research and the Program of the Institute:

Although regulation at the level of transcription is currently thought to be the primary mechanism for regulating the flow of genetic information, modulation of protein synthesis and interactions between translational and transcriptional components have recently assumed increased importance. Translational regulation has been identified as a major feature of host virus interaction. Regulation of protein synthesis by hemin was once thought to be a highly specialized means of coordinating globin chain and hemin synthesis in reticulocytes; it now appears, however, to be a widespread mechanism for post-translational modulation of gene expression involving a cascade of highly specific protein kinases and other covalent modifiers. Final control of gene expression during cell differentiation, heat shock, and viral infection may also be regulated by interaction of translational components with messenger RNA, which, in turn, may interact with and be regulated by changes in the ultrastructure of the cell. It is essential, therefore, to understand the basic mechanisms involved in these processes to be able to control gene expression in the cell.

Proposed Course of the Project:

1. Polypeptides identified as components of RNA polymerase II initiation and elongation complexes will be purified from transcriptionally active nuclear extracts of HeLa and K-562 cells. Monoclonal antibodies prepared against transcription complex components will be tested for inhibition of transcriptional activity. The specific targets of these antibodies will then be purified by large scale affinity chromatography. The ultimate goal will be to assign specific functions to these transcriptional components required for the initiation process.

2. New translational and transcriptional systems are being developed to obtain *in vitro* systems which more closely approximate *in vivo* rates. Approaches being used include cell permeabilization techniques and new methods of cell disruption.

3. The effects of specific translation inhibitors and purified initiation factors on mRNA translation will be studied in thalassemic mouse reticulocyte lysate as well as virus infected cells to clarify the mechanisms involved in post-transcriptional regulation of gene expression.

4. Additional mixed oligonucleotide probes based on partial amino acid sequence data will be used to probe cDNA and genomic libraries for translation and transcription factors. The primary structure of these proteins, their genetic organization and control of their expression at both transcriptional and translational levels will be determined.

5. Binding sites for specific and general transcription factors will be defined and compared for the Adenovirus 2 major late promoter, human and mouse β and α globin genes, and SV-40 early promoter DNA. Similarities and differences among the regulatory regions of these genes will increase our understanding of how these genes are differentially regulated.

6. Once DNA binding sites for specific factors have been defined, purification by

Proposed Course of the Project (continued):

DNA affinity chromatography will be used to purify factors sufficient to perform both physical and functional studies.

Publications:

1. Tolunay, H.E., Yang, L., Anderson, W.F., and Safer, B.: Isolation of an active transcription initiation complex from HeLa cell-free extract. Proc. Natl. Acad. Sci. USA 81, 5916-5920 (1984).

2. Safer, B., Yang, L., Tolunay, H.E., and Anderson, W.F.: Isolation of stable preinitiation, initiation, and elongation complexes from RNA polymerase II-directed transcription. Proc. Natl. Acad. Sci. USA 82, 2632-2636 (1985).

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5. Krieg, T.M., Schafer, M.P., Cheng, C.K., Filpula, D., Flaherty, P., Steinert, P.M., and Roop, D.R.: Organization of a type I keratin gene. J. Biol. Chem. 260, 5867-5870 (1985).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02214-08 MH

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Cloning of Eukaryotic Globin Gene Sequences

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Patricia E. Berg	Senior Staff Fellow	LMH, NHLBI
Others:	W. French Anderson	Chief	LMH, NHLBI
	Yu Gong	Visiting Fellow	LMH, NHLBI
	Donna Williams	Postdoctoral Fellow	LMH, NHLBI
	Rebecca King	Medical Technologist	LMH, NHLBI
	Kathy Stambaugh	Biol. Lab. Technician	LMH, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Hematology

SECTION

Section on Molecular Cloning

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.7	3.1	1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to use the mouse β-globin system as a model for eventual gene therapy of β-thalassemia in humans. We have used recombinant DNA technology to study two areas important for proper regulation of a gene introduced into cells: a) the effect of DNA enhancing sequences on the mouse β-globin promoter, both in tissue culture cells and in mice; and b) possible mechanism of enhancer function. To test the effect of known enhancers and to look for new enhancers, various plasmids, called expression vectors, have been constructed. Attempts to isolate proteins which influence enhancer activity are also underway.

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Project Description:

Objectives: There are three objectives: (1) to study the effect of known enhancer sequences on expression from the mouse β-major globin gene promoter in order to maximize gene expression and examine the specificity of enhancers; (2) to detect new enhancers in order to test their effects on the mouse β-globin promoter and their cell type specificity; and (3) to try to understand how enhancers function.

Methods Employed:

1. Galactokinase activity is assayed using a transient expression assay. This involves introducing plasmid DNA into monkey kidney cells (CV-1 cells), mouse cells (L cells), Chinese hamster cells (CH cells) or mouse erythroleukemia cells (MELC) in culture, then assaying galactokinase by starch gel electrophoresis, in vitro tube assays, or Western blots.

2. Three different plasmids have been used to stably transform a galK negative mutant of CH cells, R1610. These plasmids include one without an enhancer, one with the SV40 enhancer and one with the HaSV enhancer. Cell extracts have been analyzed for galactokinase activity and plasmid DNA copy number was determined by DNA hybridization. In addition, assays are being performed to determine the degree of methylation of the plasmid DNA and its chromatin conformation.

3. Stable transformants of mouse erythroleukemia cells (MELC) have been isolated containing the same plasmid DNAs described in (2). These are being tested not only using the techniques described in (2), but also testing the effects of induction of the MELC on plasmid expression since the mouse β-globin promoter is activated under these conditions.

4. In collaboration with Dr. Grace Ju of the Roche Institute for Molecular Biology, we tested for the presence of enhancer DNA in the Rous sarcoma virus (RSV) long terminal repeat (LTR) DNA using an enhancer screening system which we have developed. Different segments of the LTR were cloned into our expression vector containing the β-globin promoter and assayed using transient expression in quail cells, CV-1 cells and CH cells.

5. In order to look for a mouse specific enhancer, DNA from the LTR region of a mouse specific virus, the spleen focus forming virus (SFFV), has also been cloned into our expression vector in collaboration with Drs. Sandra Ruscetti and Linda Wolfe, NCI. This virus specifically infects erythroid cells so that enhancer activity, if present, may be highest in MELC.

6. In collaboration with Sheldon Goldberg in this laboratory, we have assayed a DNA sequence inserted into a β-thalassemic mouse β^{maj}-globin gene locus for its biological activity using our expression vector. Galactokinase assays have been performed on cell extracts after transient assays and from stable transformants.

7. In collaboration with Dr. Keith Humphries, NHLBI, plasmid DNA has been microinjected into fertilized mouse oocytes which were then implanted in pseudopregnant females. The presence of the plasmid was determined by Southern blotting of DNA

Methods Employed (continued):

from the tails of the offspring. Galactokinase activity was assayed in various tissues before and after treatment with chemicals such as 5-azacytidine which is known to activate certain cloned genes.

8. A possible mechanism of enhancer activity is being studied using computer analysis and cloning in a prokaryotic vector designed to detect transcriptional termination. Transcriptional termination is measured in E. coli cell extracts using galactokinase assays. The site of termination is determined by S1 nuclease analysis of cellular RNA.

9. We are attempting to identify and isolate protein factors which bind to enhancer DNA using proteins fractionated from HeLa cells and a plasmid with multiple copies of the SV40 enhancer. Binding to the ^{32}P-labelled DNA is determined by a filter binding assay.

Major Findings:

1. Stable transformants of CH cells containing the galK plasmids with and without enhancers have been analyzed to determine enhancer function when integrated into host cell DNA. At copy numbers of four or lower, there is some enhancer activity, but at higher copy numbers the galK gene becomes progressively less active, so enhancer function cannot be assessed. Activity is largely independent of integration site. The inactivation of galK does not appear to be due to DNA methylation; chromatin studies are in progress.

2. An enhancer was defined in the RSV LTR which activates both the mouse β-globin promoter and retroviral LTR promoters. There was a correlation between the amount of activation and the distance between the promoter and enhancer elements.

3. A putative enhancer from SFFV has been cloned into our expression vector. It shows low, but significant, enhancing ability in mouse L cells and NIH 3T3 cells. Assays in MELC are in progress.

4. Assays for activity on the DNA inserted in the mouse β^{maj}-globin gene (see Section 6 above) have shown this DNA does enhance transcription of the mouse β^{maj}-promoter when tested in CH cells, both in transient assays and using stable transformants.

5. After microinjection, 16 mice were born, 2 of which were positive for the presence of the expression vector plasmid DNA. One died at birth and the other (a male) was mated with wild type females. Germ line transmission of plasmid DNA has been observed to the fourth generation, with Mendelian segregation. Various tissues containing the plasmid DNA were negative when tested for galactokinase activity but were found to have heavily methylated DNA. Partial demethylation of this DNA was achieved by injecting the mice with a chemical, 5-azacytidine. Galactokinase levels increased only slightly with this treatment.

6. Computer analysis has identified possible DNA secondary structures for enhancers. We have developed a simple in vivo assay in E. coli based on the

Major Findings (continued):

transcriptional termination we have observed with enhancers which will allow us to easily identify enhancer mutants. These studies are now in progress.

7. Filter binding assays have permitted identification of a protein fraction containing binding activity for the SV40 enhancer. This fraction is being further purified.

Significance to Biomedical Research and the Program of the Institute:

The experiments described here should increase our understanding of gene expression both at the molecular level and the organismic level. The effect of enhancer sequences on cell and tissue specific expression from the mouse β-globin promoter will be studied using known enhancers and enhancers we identify in our screening system. Identification of cellular proteins which bind to enhancer DNA will allow us to develop an in vitro system to study enhancers at the molecular level. The combination of these two approaches, in vivo and in vitro, should yield valuable information concerning regulation of expression of the mouse β-globin promoter. This knowledge will be valuable in treatment of the β-thalassemic mouse and, eventually, in gene therapy of humans.

Proposed Course of the Project:

Studies to understand enhancer function on a molecular level will continue, with an emphasis on finding cellular factors which bind to enhancers, in order to better understand eukaryotic gene regulation in general and β-globin regulation specifically. In addition, possible mouse specific enhancers will be tested in our screening system. Such an enhancer, if found, would be tested by microinjection of an expression vector containing this enhancer into mouse oocytes to look for tissue specific gene expression.

Publications:

1. Berg, P.E. and Anderson, W.F. Correlation of gene expression and transformation frequency with the presence of an enhancing sequence in the transforming DNA. Mol. Cell. Biol. 4: 368-370, 1984.

2. Huberman, M., Berg, P.E., Curcio, M.J., DiPietro, J., Henderson, A.S., and Anderson, W.F. Fate and structure of DNA microinjected into mouse L TK$^-$ cells. Exp. Cell Res. 153: 347-362, 1984.

3. Berg, P.E., Popovic, Z., and Anderson, W.F. Promoter dependence of enhancer activity. Mol. Cell. Biol. 4: 1664-1668, 1984.

4. Humphries, R.K., Berg, P.E., DiPietro, J., Bernstein, S., Baur, A., Nienhuis, A.W., and Anderson, W.F. Transfer of human and murine globin-gene sequences into transgenic mice. Am. J. Human Genet. 37: 295-310, 1985.

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Publications (continued):

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6. Anderson, W.F., Goldberg, S., Kantoff, P., Berg, P., Eglitis, M., and Humphries, R.K. Attempts at gene therapy in β-thalassemic mice, in Fifth Cooley's Anemia Symposium (Bank, A., Anderson, W.F., and Zaino, E.C., eds.), Ann. NY Acad. Sci. 445, 445-451, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02216-06 MH

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Correction of Genetic Defects by Gene Transfer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. French Anderson	Chief	LMH, NHLBI
Others:	Philip Kantoff	Medical Staff Fellow	LMH, NHLBI
	Sheldon Goldberg	Medical Staff Fellow	LMH, NHLBI
	David Trauber	Medical Staff Fellow	LMH, NHLBI
	Daniel Kuebbing	Senior Staff Fellow	LMH, NHLBI
	Martin Eglitis	Staff Fellow	LMH, NHLBI
	Jeanne McLachlin	Visiting Fellow	LMH, NHLBI
	Judith DiPietro	Biologist	LMH, NHLBI
	Sheri Bernstein	Biologist	LMH, NHLBI

COOPERATING UNITS *(if any)*

A. Nienhuis, CHB, NHLBI; E. Gilboa, Princeton University, Princeton, NJ; M. Blaese, MET, NCI; R. O'Reilly, Memorial Sloan-Kettering Cancer Center, New York, NY.

LAB/BRANCH

Laboratory of Molecular Hematology

SECTION

Section on Molecular Genetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.4	6.8	1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A highly efficient procedure for transferring functional genes into mammalian cells has been developed using retroviral-based vectors as a delivery system. When mouse bone marrow cells are infected in vitro with a neo-R gene and reinjected into a lethally irradiated recipient mouse, 90% of the stem cells (CFU-S) can be shown to carry an intact copy of the neo-R gene. The majority of these cells produce the neo-R gene product: phosphotransferase. Retroviral vectors containing the human gene for the enzyme adenosine deaminase (ADA) have been made and inserted into mice, monkeys, and ADA$^-$ human T and B cells. These studies are preliminary to attempting human gene therapy in patients suffering from ADA$^-$ severe combined immunodeficiency disease.

PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives: The objective of this project is to develop methods for transferring functional genes into mammalian tissue culture cells and into intact animals. The techniques, when ready, would be used for attempting to correct genetic diseases in human patients.

Methods Employed:

1. Tissue culture cells are grown under standard tissue culture conditions.

2. Plasmids containing specific cloned genes are made by standard recombinant DNA techniques.

3. Gene transfer by retroviral vectors uses standard retroviral techniques for infection and analysis.

4. Genomic mapping is carried out by standard recombinant DNA techniques.

5. Bone marrow transplantation into lethally irradiated mice and primates is by published procedures.

Major Findings:

1. A retroviral vector containing a neo^R gene can be stably integrated into the bone marrow cells of mice at a high efficiency. The neo^R gene expresses efficiently in the bone marrow and blood cells of a long-term reconstituted animal.

2. A retroviral vector containing the human ADA gene can be stably integrated into murine 3T3 cells where it expresses efficiently.

3. Primates can be transplanted with autologous bone marrow cells treated with the ADA retroviral vector in a protocol similar to the one that would be carried out for human gene therapy.

4. B and T cells from a patient suffering from ADA^- SCID can be corrected by the ADA retroviral vector *in vitro*.

Significance to Biomedical Research and the Program of the Institute:

The long-term aim of much of the work in molecular genetics is to develop techniques for treating or curing human genetic defects. This project utilizes recombinant DNA technology, retrovirology, mouse genetics, embryology, and cell biology techniques to try to accomplish this goal.

Proposed Course of the Project:

The primate and murine bone marrow transplant model systems will continue to be developed in order to insure that ADA can be expressed efficiently in the bone marrow and blood cells of long-term reconstituted animals. The safety of the

Proposed Course of the Project (continued):

procedure will be analyzed in detail in preparation for carrying out the same procedure in patients with ADA^- SCID.

Publications:

1. Chiang, Y.L., Ley, T.J., Sanders-Haigh, L. and Anderson, W.F.: Human globin gene expression in hybrid 2S MEL X human fibroblast cells. Somat. Cell Molec. Genet. 10: 399-407, 1984.

2. Ley, T.J., Chiang, Y.L., Haidaris, D., Anagnou, N.P., Wilson, V.L. and Anderson, W.F.: DNA methylation and regulation of the human β-globin-like genes in mouse erythroleukemia cells containing human chromosome 11. Proc. Natl. Acad. Sci. USA 81: 6618-6622, 1984.

3. Anderson, W.F.: Prospects for human gene therapy. Science 226: 401-409, 1984.

4. Eglitis, M.A., Kantoff, P., Gilboa, E. and Anderson, W.F.: Gene expression in mice following high efficiency retroviral-mediated gene transfer. Science, 1985 (in press).

Annual Report of the
Section on Laboratory Animal Medicine and Surgery, Surgery Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1985

The Section functions primarily in a support role to all laboratories of IR providing care for many species of animals, technical assistance in preparation and maintenance of animal models for various experimental regimens, and the development of animal resources not otherwise available.

Maintenance of various small animal species has been accomplished in designated areas in close proximity to IR laboratories in Buildings 3, 10, and 36. Large animal species are maintained in Buildings 3, 28, the NIHAC, and at Luray, Virginia. Postoperative intensive care and treatment of surgery patients is completed in Buildings 3, 14-E, and 28.

The animal surgery laboratory located in Building 14-E supported studies for investigative staff in the Cardiology Branch, Clinical Hematology Branch, Hypertension-Endocrine Branch, Laboratory of Chemistry, Laboratory of Kidney and Electrolyte Metabolism, Laboratory of Technical Development, Molecular Disease Branch, and the Surgery Branch in preparation of experimental animal models, completing cardiovascular studies and in collecting various biological specimens. The laboratory operates an x-ray catheterization suite, blood analysis laboratory, sterile operating suites, and special study suites required to meet IR requirements.

The NHLBI sheep colony continues year-round breeding of laboratory sheep. More than 475 animals were delivered to laboratories meeting requirements of gestation stages from 120-140 days and various age and size lambs, young adults, and aged sheep. In addition, postoperative animal models have been maintained at the colony, 10-15 units of blood has been delivered every 2-3 weeks for use in cardiopulmonary by-pass studies and more than 30 tons of feed supplies have been delivered to NIH to allow continued feeding of similar feed rations to sheep maintained for biomedical research studies.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03401-09 LAMS

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Newfoundland Breeding Colony

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. E. Pierce	Chief	SLAMS, SB, DIR, NHLBI
Others:	M. Jones	Senior Surgeon	SB, NHLBI

COOPERATING UNITS *(if any)*

Clinic of Surgery Z01 HL 02697-06 SU

LAB/BRANCH

Surgery Branch

SECTION

Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Newfoundland Breeding Colony has been developed as a source of laboratory Dogs affected with left ventricular hypertrophy (LVH) due to infracoronary left ventricle outflow tract obstruction and other spontaneously occuring heart defects. All have some form of hereditary subaortic stenosis (SAS) and/or pulmonary outflow tract obstruction.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description:

Availability of a naturally occurring animal model for study of LVH resultant of infracoronary LV outflow tract obstruction is important because technical difficulties have not been satisfactorily overcome in attempts to produce such a defect in normal animals.

Discrete subaortic stenosis has been studied in Newfoundland dogs at the School of Veterinary Medicine, University of Pennsylvania where initial breeding experiments suggested that it is inherited and either a polygenic or an autosomal dominant trait with modifiers.

More than 60 dogs have been maintained with monitoring of hemodynamics using cardiac catherazation and ultrasound techniques.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03402-09 LAMS

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

NHLBI Laboratory Sheep Colony

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Joseph E. Pierce, D.V.M., Chief, SLAMS, SB, DIR, NHLBI

COOPERATING UNITS *(if any)*

1. Laboratory of Developmental Neurobiology, IRP, NICHD
2. VRB, DRS

LAB/BRANCH

Surgery Branch

SECTION

Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The Laboratory Sheep Colony is an NIH animal resource providing varied age sheep that meet specific year-round requirements of the Laboratory of Kidney and Electrolyte Metabolism, Laboratory of Technical Development and the Surgery Branch, DIR, NHLBI; and the Laboratory of Developmental Neurobiology, IRP, NICHD. Maintenance regimens in use have resulted in successful year-round breeding and production of healthy varied age sheep.

Practices that have contributed to reduction of undesired seasonal variables include: (1) continuous prophylactic immunization of all age animal groups; (2) accurate pregnancy diagnosis during first trimester using Doppler ultrasound; (3) monitoring of animal health using various diagnostic laboratory techniques; and (4) many husbandry techniques unique to this colony. Such practices have been cost prohibitive in commercial sheep flocks that result in inconsistent availability and existence of varied states of health in animals delivered for laboratory use.

7 y 8

Project Description:

The breeding colony continues as a source of sheep for the Surgery Branch, Laboratory of Technical Development, and Laboratory of Kidney and Electrolyte Metabolism meeting requirements of young lambs and pregnant ewes as required. From 730 to 850 animals were maintained to allow delivery of more than 550 sheep to NIH and other facilities for laboratory use during the report period.

The contractor has been responsible for developing and updating husbandry techniques as instructed by the project officer that allow optimal conditions for natural year-round breeding at the contract site. The project officer has been responsible for monitoring techniques and updating guidelines followed by the contractor to meet NIH laboratory requirements along with animal health groups to common sheep diseases.

Immunization protocols direct personnel to administer specific toxoids and bacterins to lambs with bi/tri-weekly boosters of each by the 6th week of age. This practice has essentially eliminated enterotoxemia and more than 90% of chronic pneumonia previously experienced. Repeated administration of indicated biologicals is carried out in all age groups at designated periods of development and production.

The necessity of an accurate method of pregnancy diagnosis during early gestation was determined during initial development of the colony. Natural seasonal and environmental conditions effect the conception rate which varies from 30-100% per month. Lindahl's technique using Doppler ultrasound with rectal examination has been adequate. It allows accurate diagnosis of up to 100% of pregnant sheep from 21-35 days gestation. Examinations are performed weekly by contract personnel with more than 1,500 examinations completed per year to detect approximately 500 pregnant ewes conceived over a 52-week period.

Laboratory tests are continuously performed to monitor flock health. Contract personnel monitor internal parasite infestation by random fecal sampling from various animal groups with microscopic examination using direct and flotation methods. CBC and blood chemistry profiles are performed on animals delivered to the laboratory to monitor health and nutrition status. Microbiological and serological screening for detection of suspected disease entities is carried out when indicated.

Also, 10-20 units of blood has been collected in CPD bags every 2-3 weeks for use in cardiopulmonary by-pass studies in NHLBI laboratories.

More than 30 tons of feed supplies were delivered to NIH to allow continuation of colony animal diets in laboratory facilities.

This project will continue as IR and other NIH programs have sufficient requirements that justify the continued support of this laboratory sheep resource. Production goals and total numbers of animals maintained will be varied as required by changing demands of laboratories.

Contract Information

Contract Number:	263-80-C-0007 - approximately $375,000 - 10/1/84-12/31/85
Contract Site:	White House Farms, Inc. Rt. 1, Box 403-E Luray, Virginia 22835
PI:	Max Foltz, Contractor Rick Miller, Colony Manager
Total Manyears:	8.0
Professional:	2.0
Other:	6.00

ANNUAL REPORT OF THE
FORMER SECTION ON THEORETICAL BIOPHYSICS
OFFICE OF THE DIRECTOR OF INTRAMURAL RESEARCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1984 through September 30, 1985

This is a report of certain specified activities within the Office of the Director, Intramural Research, NHLBI. The primary purpose of these activities has been the extension of the theory of transport processes in biological systems, with particular reference to problems in cardiovascular, renal and membrane physiology. The main concern has been both with the formulation of theoretical models and with the development of mathematical and computational methods for their analysis. Much of this research has been centered on the mechanism of urine formation in the mammalian kidney and on the theoretical aspects of solute and water movement in tubular structures. A second activity has been aimed at understanding underlying physical processes in nuclear magnetic resonance (NMR) spectroscopy.

During the past year, areas of investigation have included: (1) The qualitative analysis of equations describing kidney models that include chemical reactions, (2) the development of analytical solutions of models of isolated tubules, (3) the development and analysis of numerical methods for solution of more complex models, (4) computer simulations, (5) solution of the Bloch equations in NMR spectroscopy.

In earlier studies it was found that the solution manifolds of kidney models have multiple solution branches; research on this problem has continued. The solution of a multinephron, multisolute model of the mammalian kidney by Newton and continuation methods (Mejia, R. and J.L. Stephenson, Math. Biosciences 68:279-298(1984)) was used previously to obtain a connected component of the steady state solution manifold. A conversational path-follower for systems of nonlinear equations has now been developed (R. Mejia, J. Comput. Phys., to appear) and used to solve a model that includes multiple nephrons that discharge into a common collecting duct and the renal pelvis. Results of studies performed on the effect of various parameter modifications on the concentrating mechanmism are in preparation for publication (Kaimal, Mejia and Stephenson).

A detailed study of the solutions of a four tube model of the renal medulla (J.B. Garner, R.B Kellogg and J.L. Stephenson, Math. Biosciences 65:125-150(1983)) showed that the solution manifold is folded into three sheets. The upper sheet consists of highly concentrating solutions to the model equations, and on this sheet the concentrating ratio is determined by the dissipative loss of solute caused by inefficient vascular exchange. With no loss, the upper sheet is not present, and the solution manifold contains at most two sheets. An analytic model for a limiting form of the model equations suggests the existence of solutions bifurcating from the upper stable sheet. The conversational path-follower has been used to test this hypothesis using a four tube model with a range of membrane parameters.

No bifurcating solutions have been identified although the solution sheet is highly convoluted, especially as a function of the volume flow entering the descending portion of Henle's limb (Kellogg and Mejia).

It is widely accepted that bicarbonate reabsorption in the mammalian proximal tubule is mediated by proton secretion. The renal cortical collecting duct is also capable of simultaneous proton secretion and bicarbonate secretion. Studies with isolated perfused cortical collecting ducts have shown that the luminal pH cannot be predicted from the luminal bicarbonate concentration because proton secretion generates a disequilibrium pH in the lumen. That is, proton secretion lowers the pH by reacting with bicarbonate to form carbonic acid. Consequently, carbonic acid accumulates to a concentration greater than the equilibrium concentration determined by the dissolved CO2 concentration. A disequilibrium pH in the lumen is physiologically important because it increases the osmotic gradient for the diffusional entry of ammonia, an important urinary buffer. In order to study this quantitatively, equations for the concentration of total CO2 (i.e., CO2, HCO3- and H2CO3) and H+ concentration in a tubule have been postulated. A steady state model has been solved analytically (R. Mejia, Proc. 1984 MACSYMA Users' Conference, 35-37(1984)) using computer algebra to obtain radial concentration profiles. In the steady state, a solution for the radial concentration of the individual species: CO2, HCO3-, H2CO3, and H+ has been obtained numerically, and a two dimensional model describing radial and axial profiles is being developed (Mejia, Knepper and Star).

The nonlinear differential equations that govern the temporal response of the magnetization in the presence of an irradiating magnetic field are known as the Bloch equations. In order to better understand phenomena observed in NMR spectrography an analysis of the Bloch equations has been undertaken. Numerical solutions have been obtained using the conversational path-follower noted above. The primary interest has been an appropriate normalization for a singular perturbation analysis. A second area of current investigation is the application of the maximum entropy principle to image reconstruction in Fourier transform NMR (Mejia, Ferretti and Weiss)

Annual Report of the Pathology Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1984 to September 30, 1985

Studies during this time period involved coronary, valvular, congenital, and miscellaneous heart disease in human beings and the Ultrastructure Section of this Branch was involved in a number of studies involving experimental animals.

CORONARY ARTERY DISEASE

One study involved 78 necropsy patients with transmural acute myocardial infarction to correlate their mode of death, the interval between onset of infarction and death, and the presence or absence of coronary thrombus with the extent of the infarct. Infarct size was assessed quantitatively as a percent of total left ventricular mass. Infarct size had not been correlated previously with these various factors. The mean interval betwen the onset of acute infarction and death was 12 $\pm$ 13 days. Infarct size averaged 23 $\pm$ 14% of the left ventricular mass. Patients who died in cardiogenic shock had the largest infarcts (13 $\pm$ 11%) and those dying of cardiac ruputure had the smallest infarcts (15 $\pm$ 9%) and the shortest interval between onset of infarction and death (7 $\pm$ 8 days). Coronary thrombi were present in 58 patients (74%). When present, thrombus was observed in the coronary artery which had supplied the infarct area and was superimposed on advanced artherosclerotic plaque. There was no relation between the extent of luminal obstruction by thrombus and infarct size. The absence of coronary thrombus at necropsy was associated with either small infarcts or prolonged survival following infarction.

Little information is available on the morphologic features of hearts in patients with ischemic cardiomyopathy. We examined the hearts of 81 necropsy patients (95% men) with severe congestive heart failure of greater than 3 months duration, left ventricular transmural scar, and greater than 75% cross-sectional area narrowing by atherosclerotic plaque of one or more of the 4 major epicardial coronary arteries. The hearts in all 81 patients were heavier than normal (mean 585 g.). Left and/or right ventricular thrombi occurred in 37 patients (46%), only 4 had systemic emboli; of the 44 patients without intercardiac thrombi, none had emboli. The severity of coronary narrowing was variable. In 24 patients (30%) only one artery was narrowed greater than 75% in cross-sectional area; in 22 patients (27%), 2 arteries were so narrowed; in 32 patients (39%), 3 arteries, and in 3 patients (4%), 4 arteries were so narrowed. The size of the left ventricular scar also varied. Of the 81 patients, 58 (72%) had large scars; 10 (12%) had moderate size scars, and 13 had small scars. The size of the left ventricular scar correlated with a history of habitual alcoholism: of the 16 habitual alcoholics, 6 had small and 8 had large scars; of the 65 non-alcoholics, 7 had

small scars and 50 had large scars. The chronic congestive heart failure in the 68 patients with either moderate or large size scars is readily attributed to the left ventricular damage; in the 13 patients with small left ventricular scars, however, the chronic heart failure more reasonably may be attributed to another factor, e.g., alcoholism, despite coronary arterial narrowing, similar in severity to that in patients with large ventricular scars.

We also studied at necropsy the hearts of 18 patients who during life had had chronic congestive heart failure of greater than 3 months duration, greater than 75% cross-sectional area narrowing of 1 or more of the 4 major epicardial coronary arteries by atherosclerotic plaque, but no left ventricular fibrosis or necrosis at necropsy. Because grossly visible myocardial lesions in them were absent, the severe chronic congestive heart failure in these 18 patients cannot reasonably be attributed to coronary artery disease. It is most reasonable to believe that this group of patients had idiopathic dilated cardiomyopathy and that the coronary artery disease was coincidental.

To assess the status of a saphenous vein excised for coronary bypass grafting, 3,394 cm of remnant saphenous vein from 402 patients who underwent bypass coronary surgery were examined. The saphenous vein remnants were 0.5 to 52 cm long, (mean 8.4). They were sectioned into 5-mm long segments, and the resulting 6,788 5-mm segments were examined histologically: 5,896 (87%) were narrowed 0 to 25% in cross-sectional area by fibrous tissue; 853 (12%) were narrowed 26 to 50%; 23 (0.6%), 51 to 75%, and 16 (0.4%) segments were narrowed 76 to 100%. Of the 16 segments severely narrowed, 7 (44%) were nearly totally occluded by fibrous tissue. In 17 patients who died within 24 hours of coronary artery bypass graft, similar degrees of luminal narrowing were observed in remnant segments and in utilized segments of saphenous vein. Thus, significant preexisting luminal narrowing of saphenous vein used for coronary artery bypass graft is infrequent. The intimal fibrous thickening is variable within the same vein when each 5-mm-long segment is analyzed; it is variable from 1 vein to another in the same patient, and it varies among patients.

VALVULAR HEART DISEASE

We described certain electrocardiographic findings in 30 necropsy patients with clinically isolated pure, chronic, severe aortic regurgitation. They were 19 to 65 years old (mean 45). The hearts of the 22 men ranged in weight from 430 to 1,110 g (mean 717) and of the 8 women, from 375 to 950 g (mean 638). Four had grossly visible left ventricular scars. All but 1 patient was in sinus rhythm. The PR interval was >0.20 second in 8 patients (28%) and the QRS duration was $\geq$ 0.12 second in 6 patients (20%). Only 5 patients (17%) had 1 or more ventricular premature complexes recorded on the resting electrocardiogram analyzed. The mean QRS amplitude for each of the 12 leads average 23 mm. The highest mean QRS voltage occurred in leads V_2 and V_3 (each 38 mm), and the lowest in lead aVR (11 mm). The mean QRS voltage in V_5 was higher than V_6 (33 vs 28 mm) and in 22 patients (73%) the QRS voltage in V_5 was higher than V_6. The sum of the S wave in V_1 plus the larger of the R wave in V_5 or V_6 (Sokolow-Lyon index) averaged 51

mm and in only 22 patients (73%) was it >35 mm.

Little detailed information is availablee on the structure of the normal aortic valve. We measured the area, weight and 4 linear variable in each aortic valve cusp in 100 necropsy patients with normally functioning aortic valves, the volume of each sinus of Valsalva and the aortic area at the sinotubular junction in the same patients. The sums of the aortic valve cuspal areas, cuspal weights and sinus of Valsalva volumes increased with age ($p < 0.001$) and with heart weight ($p < 0.001$). All 3 variables (cuspal area, cuspal weight and sinue of Valsalva volume) also increased with age and heart weight relative to each other. The luminal area of aorta at the sinotubular junction also increased with age and heart weight and it also increased as the sum of the aortic valve cuspal areas and weights and sinus of Valsalva volumes increased. In only 16% of the 100 patients were the 3 aortic valve cusps of similar size (less than 5% difference in area between cusps); in 51%, 1 cusp was of different size than the other 2, and in 33% of patients all 3 cusps were of different sizes.

Hearts weighing over 1000 g are rarely observed in human beings. During a 25 year period, we collected the hearts in 23 patients in whom the heart at necropsy weighed at least 1,000 g. (mean 1,106). Certain clinical and morphologic features in the 23 patients were described. The heart weight to body weight ratio ranged from 1.2 to 2.7 (normal 0.40). The 23 patients were derived from examination of the hearts of 7,671 patients with various cardiovascular disorders over a 25-year period. The massive cardiomegaly was the result of aortic regurgitation in 14 patients (61%): isolated in 8, associated with mitral regurgitation in 4, and with ventricular septal defect in 2. Three others (13%) had combined aortic valve stenosis and aortic regurgitation and 1 patient (4%) had mitral stenosis and regurgitation and mild aortic stenosis. Four patients (17%) had hypertrophic cariomyopathy, and 1 patient (4%) had ventricular septal defect with mitral stenosis. They were 20 to 64 years old (mean 442) and 21 (91%) were men. Four patients at necropsy had 1 or more major coronary arteries narrowed more than 75% in cross-sectional area by atherosclerotic plaque, and only 4 patients had grossly visible left ventricular scars, 2 of whom had insignificant coronary narrowing. Examination of electrocardiograms in 17 the 23 patients disclosed that Sokolow-Lyon criteria for left ventricular hypertrophy was achieved in only 12 patients (71%) and Romhilt-Holt QRS voltage criteria faired even worse. Total 12-lead QRS voltage was more than 175 mm (10 mm = 1mV) in 16 patients (94%) and it was more than 250 mm in 13 patients (76%). Total 12-lead QRS voltage in 17 patients ranged fron 140 to 601 mm (mean 323). Measurement of the sum in diagnosing left ventricular hypertrophy by electrocardiogram.

CONGENITAL HEART DISEASE

A long interest of this Branch has been examination of hearts at necropsy in patients who have died suddenly. We have found through the years that various congenital coronary anomalies may lead to sudden death. In one study in 1984 we described 5 patients at necropsy who were found to have origin of the left main coronary artery from the right sinus of Valsalva with coursing

of the left main to the left side of the heart between the aorta and pulmonary trunk. Three (all boys) of the 5 patients died suddenly at 13, 14, and 19 years, respectively. Two of them had had one or more episodes of syncope and the third had an abnormal electrocardiogram. The fourth patient, a 64-year old woman, died of chronic congestive heart failure 1 year after an acute myocardial infarction. She had insignificant coronary atherosclerosis. The fifth patient, an 81-year-old man, died of chronic alcoholism, having been free of symptoms of cardiac dysfunction during life. Additionally, clinical and necropsy findings were summarized in 38 previously report necropsy patients with the coronary anomaly. Of the 38 (34 males [89%]), 23 (61%) died suddenly in the first 2 decades of life; death in 6 (16%) others appears to have been related to coronary atherosclerosis nd 9 patients (24%) died from non-coronary causes. Thus, this anomaly is life threatening. Why it frequently causes fatal cardiac arrest in some young individuals and allows a normal span in others remains unclear.

MISCELLANEOUS

Anorexia nervosa is a condition which has received considerable attention in the lay press in recent years. Little morphologic information is available on patients with fatal anorexia nervosa. It is known that these individuals often have ventricular arrhythmias. We examined at necropsy the hearts of 3 women with fatal anorexia nervosa. Necropsy examination failed to establish an anatomic cause of death. Electrocardiograms recorded 7 days or less before death showed various degrees of Q-T interval prolongations: Q-T intervals corrected for heart rate measured 0.61 s, 0.47 s, and 0.46 s, respectively. Terminal ventricular tachyarrhythmias were documented in 2 patients, including torsade de pointes in 1. The necropsy and clinical findings in these 3 cases provide evidence that sudden death in anorexia nervosa, like sudden death in liquid-protein dieting, may result from ventricular tachyarrhythmias related to Q-T interval prolongation. For such patients, electrocardiographic monitoring should be routine.

MORPHOLOGIC STUDIES IN EXPERIMENTAL ANIMALS

The Ultrastructure Section of this Branch has long been interested in cardiac changes produced by cardiotoxicity from various drugs. Cardiac changes were studied in a miniature swine who had received minoxidil. Two types of lesions were observed: hemorrhagic ones related to drug-induced vascular injury and papillary muscle necrosis related to ischemic injury from hypoperfusion during minoxidil-induced tachycardia and hypotension. These studies were done in conjunction with the Division of Drug Biology, Food and Drug Administration.

The potential of N-acetylctsteine (a free radical scavenger) and ICRF-187 ((±-)1,2-bis(3,5-dioxopieperazinyl-1yl) propane), alone and in combination, to protect against chronic doxorubicin cardiotoxicity was examined in beagle dogs. This cardiotoxicity was prevented by pretreatment of the animals with ICRF-187, but not be pretreatment with N-acetylcysteine.

Poikilocytosis was observed in peripheral blood smears obtained from dogs given doxorubicin. Concurrent administration of thyroxine and ICRF-187 did not prevent the occurrence of poikilocytosis in doxorubicin-treated dogs. Administration of N-acetylcysteine with doxorubicin resulted in a mild increase in the extent of poikilocytosis compared with that observed in dogs given doxorubicin alone. The hematologic changes were not accompanied by adverse clinical signs referable to the doxorubicin-induced alterations in erythrocytes.

A comparison was made of the severity of chronic doxorubicin cardiotoxicity in adult male spontaneously hypertensive rates and in genetically related normotensive Wistar-Kyoto rats. Spontaneous hypertensive rats were found to be much more sensitive than normotensive rats to the cardiotoxic effects of doxorubicin.

Comparisons were made of the protective activity of ICRF-187 and a series of related bis-dioxopiperazine analogues against the acute toxicity produced by daunomycin (daunorubicin) in Syrian golden hamsters. The protective activity was found not to be stereospecific with respect to the l- and the d-isomers of ICRF-159, namely, ICRF-186 and ICRF-187. Protection was minimal or absent when the hamsters were pretreated with various doses of ICRF analogues in which slight changes had been made in the dioxopiperazine rings or in the central chain. Thus, very little alteration can occur in the basic structure of ICRF-187 without loss of its protective activity against anthracycline cardiotoxicity.

Comparisons were made of the morphological characteristics of endocardial lesions produced by transcatheter delivery of either laser irradiation or electrical shock in closed-chest anesthetized dogs. The results obtained show that transcatheter laser photoablation can produce controlled endocardial lesions with less energy and fewer deleterious effects than transcatheter electrode shock.

Methods of light microscopy, transmission electron microscopy and immunohistochemistry were used to study the morphogenesis of elastic fibers in nuchal ligament, aorta and lung of fetal, neonatal and postnatal sheep. Developing elastic fibers were found to consist of amorphous components (elastin) and surrounding microfibrils. Antielastin antibody reacted with the microfibrils and with amorphous components. Microfibrils appear to serve as sites for aggregation of elastin during elastogenesis. The process of maturation of elastic fibers can be evaluated by the use of appropriate staining methods, including the periodic acid-silver methenamine technique and the tannic acid method of Kajikawa.

The patterns of evolution of pulmonary structural remodeling after experimental paraquat toxicity were studied in cynomolgous monkeys given 1 or 2 injections of 10 mg/kg of paraquat and sacrificed 2 days to 8 weeks later. Intraalveolar fibrosis was found to be more important than interstitial fibrosis in the structural remodeling that occurs in paraquat lung, because it results in obliteration of alveoli, coalescence of alveolar walls and loss of functional alveolar-cappillary units.

Morphologic and immunohistochemical studies were made of open lung biopsies from patients with histiocytosis X to detect the histiocytosis X cells that are typical of this disorder. These cells were specifically labelled by OXT6 monoclonal antibody and by antibodies against S-100 protein. The diagnostic applications of these techniques are discussed in detail.

Autoradiographic studies were made on enzymatically dissociated atrial myocytes to examine the DNA synthetic response of atrial myocardium to left ventricular infarction. These studies showed that increased DNA synthesis, manifested as increased labelling of myocytes with tritiated thymidine, occurs in atrial myocardium as a response to left ventricular infarction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03849-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Acute cardiotoxicity of minoxidil in miniature swine

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
John F. van Vleet, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana
Eugene H. Herman, Division of Drug Biology, Food and Drug Administration, Washington, D. C.

COOPERATING UNITS *(if any)*

School of Veterinary Medicine, Purdue University, West Lafayette, Indiana
Division of Drug Biology, Food and Drug Administration, Washington, D.C.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Histologic and ultrastructural studies were made of the cardiac morphologic alterations induced cutely in miniature swine by the administration of minoxidil, a vasodilating antihypertensive agent. Two types of lesions were observed: 1) hemorrhagic lesions, related to drug-induced vascular injury and localized in epicardial and subepicardial arterioles, and 2) papillary muscle necroses, related to ischemic injury from hypoperfusion during minoxidil-induced tachycardia and hypotension.

759

GPO 914-918

Project Description

Objectives: To define the light and electron microscopic features of minoxidil-induced cardiotoxicity in miniature swine.

Methods employed and major findings: Minoxidil, a vasodilating antihypertensive agent, was given orally (10 mg/kg daily for 2 days) to twelve 25- to 35-kg miniature swine. Twelve control pigs were also studied. Minoxidil-treated pigs had tachycardia and hypotension and were killed 24 hours after the second dose. Gross examination revealed diffuse hemorrhage in left atrial epicardium (n=12), and focal hemorrhages in ventricular epicardium (n=2) and endocardium (n=3). Pale areas of necrosis were observed on incision of the left ventricular papillary muscles (n=3). Acute vascular damage was associated with hemorrhage in left atrial epicardium. Affected arterioles had endothelial cell swelling and transmural and perivascular accumulations of leukocytes, edema fluid, fibrin clumps and erythrocytes. The swollen endothelial cells had large, irregularly shaped nuclei with abundant euchromatin; mitotic figures were frequent, and the cytoplasm contained numerous polysomes and cisterns of rough-surfaced endoplasmic reticulum. In the affected papillary muscles, necrotic myocytes had contraction bands, mitochondrial matrical densities, lipid accumulation, lysis of I bands, and pyknotic nuclei. The hemorrhagic lesions were thought to result from drug-induced vascular injury centered on epicardial and subepicardial arterioles, and the papillary muscle necroses were considered to be consequences of ischemic injury from hypoperfusion during minoxidil-induced tachycardia and hypotension.

Significance: These studies document the cardiotoxic potential of minoxidil and provide a partial explanation for this cardiotoxicity.

Project course: Project completed.

Publications: Van Vleet, J. F., Herman, E. H. and Ferrans, V. J.: Cardiac morphologic alterations in acute minoxidil cardiotoxicity in miniature swine. Exp. Mol. Pathol. 41: 10-25, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03850-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Effect of toxic substances on the heart

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principel Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

An extensive review was prepared of morphological aspects of the effects of toxic substances on the heart.

761

Project Description

Objectives: To review morphological changes produced in the heart by toxic substances.

Methods and major findings: In this review, the morphological reactions of the cardiovascular system to toxic injury are described under the following categories: 1) cardiac hypertrophy; 2) cardiomyopathies; 3) cardiac necroses, including infarct-like necroses produced by various agents such as sympathomimetic amines; 4) hypersensitivity (allergic) myocarditis and toxic myocarditis; 5) pericarditis, and 6) vascular changes, including hypersensitivity vasculitis, toxic vasculitis, fibromuscular hyperplasia and thromboembolism.

Significance: A wide spectrum of morphological changes has been associated with the effects of toxic drugs and chemicals on humans and experimental animals. Recognition of these changes is important from the standpoints of diagnosis and research.

Project course: Project completed.

Publications: Ferrans, V. J.: Effects of toxic substances on the heart. IN: Physiology and Pathophysiology of the Heart. Sperelakis, N. (ed.). Martinus Nijhoff, Boston, MA, 1984, pp 639-658, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03851-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Morphological aspects of myocardial lesions associated with stress

PRINCIPAL INVESTIGATOR *(List other profassional personnal below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
John F. Van Vleet, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

COOPERATING UNITS *(if any)*

School of Veterinary Medicine, Purdue University, West Lafayette, Indiana.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A survey was made of morphologic aspects of the cardiovascular lesions that develop in association with various forms of stress and of other types of alterations in the nervous system.

763

GPO 914-918

Project Description

Objectives: To review in detail morphological aspects of cardiovascular lesions associated with stress.

Methods employed and major findings: A literature review was made of cardiac morphologic changes related to various types of nervous system lesions in humans and in animals, including lesions related to: 1) various forms of stress; 2) a variety of CNS disorders such as subarachnoid and intracerebral hemorrhage, cerebral infarction, meningitis, intracranial masses; 3) electrical stimulation of different areas of the nervous system; 4) heritable disorders such as the porcine stress syndrome and the syndrome of malignant hyperthermia.

Significance: Stress produces important cardiovascular lesions, and this survey provides a comprehensive review of their morphology.

Project course: Project completed.

Publications: Ferrans, V. J. and Van Vleet, J. F.: Morphological aspects of myocardial lesions associated with stress. IN: Stress and Heart Disease, Beamish , R. E., Singal, P. K. and Dhalla, N. S., (eds.). Martinus Nijhoff Publishing, Boston, MA, 1985, pp 211-227

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03852-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of elastic fibers in fetal and postnatal sheep.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Yuh Fukuda, Visiting Expert, Ultrastructure Section, Pathology Branch, NHLBI
Ronald G. Crystal, Chief, Pulmonary Branch, NHLBI

COOPERATING UNITS *(if any)*

Pulmonary Branch, NHLBI

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Methods of light microscopy, transmission electron microscopy and immunohistochemistry were used to study the morphogenesis of elastic fibers in nuchal ligament, aorta and lung of fetal, neonatal and postnatal sheep. Developing elastic fibers were found to consist of amorphous components (elastin) and surrounding microfibrils. Antielastin antibody reacted with the microfibrils and with the amorphous components. Microfibrils appear to serve as sites for aggregation of elastin during elastogenesis. The process of maturation of elastic fibers can be evaluated by the use of appropriate staining methods, including the periodic acid-silver methenamine technique and the tannic acid method of Kajikawa.

765

Project Description

Objectives: To study the morphogenesis of elastic fibers in nuchal ligament, aorta and lung of sheep.

Methods employed and major findings: The morphogenesis of elastic fibers of nuchal ligament, aorta and lung of sheep was studied by light microscopy, transmission electron microscopy, and immunohistochemical methods for the detection of elastin. The degree of maturation of the amorphous materials of the elastic fibers was assessed morphologically in preparations stained by the tannic acid and periodic acid methenamine-silver methods. With both of these methods, the amorphous components of mature fibers stained less intensely than did those of immature fibers. Elastic fibers in early stages of development consisted of many microfibrils and a few small, branching masses of immature amorphous material. Thicker fibers were formed by the coalescence of growing masses of amorphous materials. In late stages of formation of elastic fibers, the mature amorphous materials were associated with few microfibrils, and were partially surrounded by immature amorphous materials associated with many microfibrils. Antielastin antibody reacted evenly with amorphous materials in very early stages of elastic fiber development, but reacted only with the outer zones of amorphous materials in later stages; it also reacted with the microfibrils in all stages. These findings were interpreted as indicating that the microfibrils were associated with small amounts of elastin on their surfaces. This conclusion is in agreement with ultrastructural observations showing: 1) that the development of microfibrils precedes that of the amorphous material and 2) that the microfibrils adjacent to the immature amorphous material are covered with small amounts of tannic acid-positive materials. These observations suggest that microfibrils serve as sites for elastin deposition, both in early elastogenesis and in subsequent growth of elastic fibers. However, the nature of the interaction between elastin and microfibrils remains unknown.

Significance: This study demonstrates that the degree of maturation of elastic fibers in various tissues can be assessed at the ultrastructural level by means of appropriate staining methods.

Porject course: Project completed.

Publications: Fukuda, Y., Ferrans, V. J., and Crystal, R. G.: Development of elastic fibers of nuchal ligament, aorta and lung of fetal and postnatal sheep: An ultrastructural and electron microscopic study. Am. J. Anat. 170: 597-620, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03853-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of ICRF-187 and N-acetylcysteine on chronic doxorubicin cardiotoxicity.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Eugene H. Herman, Food and Drug Administration, Washington, D. C.
C. E. Myers, Chief, Clinical Pharmacology Branch, NCI
John F. Van Vleet, School of Veterinary Medicine, Purdue University, West Lafayette Indiana.

COOPERATING UNITS *(if any)*

Food and Drug Administration, Washington, D. C.
Clinical Pharmacology Branch, NCI
School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The potential of N-acetylcysteine (a free radical scavenger) and ICRF-187 ((±)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane) , alone and in combination, to protect against chronic doxorubicin cardiotoxicity was examined in the beagle dog model. This cardiotoxicity was prevented by pretreatment of the animals with ICRF-187, but not by pretreatment with N-acetylcysteine.

767

GPO 914-918

Project Description

Objectives: To examine the potential of N-acetylcysteine (NAC) and ICRF-187, alone and in combination, to protect against chronic doxorubicin cardiotoxicity.

Methods employed and major results: Adult beagles of either sex were given 1.75 mg/kg i.v. of doxorubicin, either alone of 30 minutes after either ICRF-187 (25 mg/kg, i.p.), NAC (200 mg/kg, i.p.), or ICRF-187 (25 mg/kg, i.p.) plus NAC (200 mg/kg, i.p.) at 3 week intervals. Control dogs received similar doses of ICRF-187 and NAC but without doxorubicin. The experiment was terminated 3 weeks after the 7th injection (total doxorubicin dose, 12.25 mg/kg). The frequency and extent of myocardial lesions (vacuolization and myofibrillar loss) were assessed on a scale of 0 to 4. Such lesions were present in all 6 dogs given doxorubicin alone and were marked or severe (3 or 4+) in 5 of these dogs and moderate (2+) in 1. Lesions of comparable severity (2 to 4+) were also apparent in the hearts of dogs given the combination of NAC and doxorubicin. In contrast, no lesions (score of 0) were found in the hearts of 3 of 6 dogs given doxorubicin and ICRF-187 and in 4 of 6 dogs given doxorubicin, NAC and ICRF-187; the remaining animals in these 2 groups had minimal lesions.

Significance: The present study shows that ICRF-187 is effective and NAC is ineffective in reducing chronic doxorubicin cardiotoxicity.

Project course: Project completed.

Publications: Herman, E. H., Ferrans, V. J. , Myers, C. E. and Van VLeet, J. F.: Comparison of the effectiveness of (±)-1,2-bis(3,5-dioxopiperazinyl-1-yl)propane (ICRF-187) and N-acetylcysteine in preventing chronic doxorubicin cardiotoxicity in beagles. Cancer Res. 45: 276-281, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03854-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Toxic interactions of benzyl alcohol with bacterial endotoxin.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

Thomas A. Cebula, Food and Drug Administration, Washington, D. C.

Antoine N. El-Hage, Food and Drug Administration, Washington, D. C.

COOPERATING UNITS *(if any)*

Food and Drug Administration, Washington, D. C.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	-

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Acute toxic interactions of intravenously administered benzyl alcohol and Escherichia coli 055:B5 endotoxin were examined in rodents. The toxic effects of the combination of benzyl alcohol and bacterial endotoxin were much greater than when either of these agents was given alone. These severe effects were shown to be caused by an enhancement of the lethal properties of bacterial endotoxin.

769

Project Description

Objectives: To study the toxic interactions between benzyl alcohol and bacterial endotoxin.

Methods employed and major findings: Acute toxic interactions of intravenously administered benzyl alcohol and E. coli 055:B5 endotoxin were examined in rodents. Studies in male CD1 mice demonstrated that these agents were more toxic when given in combination than when either was administered alone. Pretreatment with 5 mg/kg diazepam protected against lethality induced by either the combination or by endotoxin, but offered little, if any, protection against the lethal effects of benzyl alcohol. Similar pretreatment with 5 mg/kg of naloxone failed to protect against either endotoxin-induced or benzyl alcohol-induced lethality, but protected significantly against the lethal effects of the combination. Male Wistar rats treated with 40 mg of benzyl alcohol showed no evidence of hepatic lesions, but rats treated in combination with sublethal doses of benzyl alcohol and endotoxin developed hepatic lesions that were more severe than those observed in rats treated only with a similar dose of endotoxin. These data show that the toxic effects produced by the combination of the two agents are due to an enhancement of the lethal properties of bacterial endotoxin.

Significance: These studies were undertaken because of evidence suggesting a role of bacterial endotoxin in the pathogenesis of sudden death in premature infants suspected of dying of toxic reactions to benzyl alcohol. The results obtained demonstrate that in vivo toxic interactions occur between benzyl alcohol and bacterial endotoxin.

Project course: Project completed.

Publications: Cebula, T. A., El-Hage, A. N. and Ferrans, V. J.: Toxic interactions of benzyl alcohol with bacterial endotoxin. Infect. Immun. 44: 91-96, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03855-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Comparison between endocardial laser photoablation and electrode schock ablation.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
E. Rene Rodriguez, Visiting Fellow, Ultrastructure Section, Pathology Branch, NLHBI
Benjamin I. Lee, Veterans Administration Medical Center, Washington, D.C.
John S. Gottdiener, Veternas Administration Medical Center, Washington, D. C.
Ross D. Fletcher, Veterans Administration Medical Center, Washington, D. C.

COOPERATING UNITS *(if any)*

Veterans Administration Medical Center, Washington, D. C.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Comparisons were made of the morphological characteristics of endocardial lesions produced by transcatheter delivery of either laser irradiation or electrical shock in closed-chest anesthetized dogs. The results obtained show that transcatheter laser photoablation can produce controlled endocardial lesions with less energy and fewer deleterious effects than transcatheter electrode shock.

771

Project Description

Objectives: To characterize and compare the effects of transcatheter laser and electrical energy on canine endocardium.

Methods employed and major findings: Thirty-five laser pulses were delivered to the endocardial surfaces of isolated canine hearts, and 33 endocardial lesions were produced by the transarterial delivery of either transcatheter laser irradiation or electrical shock in closed-chest anesthetized dogs. Laser-induced lesion dimensions in vitro and in vivo increased with increasing total dose of energy; however, the lesions produced in vivo were different in morphology and were significantly larger than lesions produced by equivalent doses of energy delivered in vitro. Endocardial lesions produced in vivo by laser at 40 and 80 J were comparable in size and morphology to those produced by electrical shock at 100 and 200 J, but transcatheter electrode shock produced significantly more ventricular tachycardia, premature beats within 7 minutes after energy discharge and wall motion abnormalities.

Significance: Laser and electrical shock ablation of endocardial tissue have been proposed for the therapy of recurrent ventricular arrhythmias. This is the first detailed comparison of the morphology of the lesions produced by the two methods under comparable conditions. The study shows that transcatheter laser photoablation can create endocardial lesions with less energy and fewer deleterious affects than transcatheter electrode shock.

Project course: Project completed.

Publications: Lee, B. I., Gottdiener, J. S., Fletcher, R. D., Rodriguez, E. R. and Ferrans, V. J.: Transcatheter ablation: comparison between laser photoablation and electrode shock ablation in the dog. Circulation 71: 579-586, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03856-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Patterns of pulmonary structural remodeling after experimental paraquat toxicity.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Yuh Fukuda, Visiting Expert, Ultrastructure Section, Pathology Branch, NHLBI
Carl I. Schoenberger, Pulmonary Barnch, NHLBI
Stephen I. Rennard, Pulmonary Branch, NHLBI
Ronald G. Crystal, Chief, Pulmonary Branch, NHLBI

COOPERATING UNITS *(if any)*

Pulmonary Branch, NHLBI

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The patterns of evolution of pulmonary structural remodeling after experimental paraquat toxicity were studied in cynomolgous monkeys given 1 or 2 injections of 10 mg/kg of paraquat and sacrificed 2 days to 8 weeks later. Intraalveolar fibrosis was found to be more important than interstitial fibrosis in the structural remodeling that occurs in paraquat lung, because it results in obliteration of alveoli, coalescence of alveolar walls and loss of functional alveolar-capillary units.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Objectives: To evaluate morphological aspects of the evolution of the interstitial and intraalveolar fibrosis produced by paraquat, a toxic herbicide.

Methods employed and major findings: Ultrastructural and immunohistochemical observations were made of the lungs of 16 cynomolgous monkeys given 1 or 2 injections of 10 mg/kg of paraquat and sacrificed 2 days to 8 weeks later. At 2-3 days, alveolar epithelial cells were denuded in many areas, and fibronectin was conspicuous in alveolar spaces. At 1 week, fibroblasts and inflammatory cells were migrating through gaps in the denuded epithelial bsement membranes, and type II alveolar epithelial cells were regenerating in some areas. At 3-4 weeks, alveoli developing intraalveolar fibrosis contained many myofibroblasts, collagen fibrils and small elastic fibers; fibrotic alveolar walls were lined by metaplastic squamous cells and bronchiolar epithelial cells. Spiraling collagen fibrils were found in interstitium but not in alveolar spaces, suggesting that they were formed from breakdown of collagen. Newly formed intraalveolar collagen was mainly of type I. At 8 weeks, intraalveolar fibrosis had led to extensive remodeling, with new gland-like alveoli lined by type II alveolar epithelial cells; alveoli without intraalveolar fibrosis had more normal architecture. Thus, intraalveolar fibrosis in paraquat lung is mediated by intraalveolar migration of interstitial cells, through gaps in the epithelial basement membranes, after epithelial injury. This is followed by connective tissue synthesis on the luminal side of the epithelial basement membrane, by differentiation of interstitial cells into myofibroblasts and smooth muscle cells, by incorporation of areas of intraalveolar fibrosis into the interstitium, and by coalescence of alveolar walls. Intraalveolar fibrosis is more important than interstitial fibrosis in the structural remodeling that occurs in paraquat lung, because it results in obliteration of alveoli, coalescence of alveolar walls, and loss of functional alveolar-capillary units.

Significance: The importance of the role of intraalveolar fibrosis in structural remodeling of the lung in fibrotic disorders has not been properly recognized. This study provides the first ultrastructural description of the mechanisms by which this type of fibrosis develops.

Proposed course: Project completed.

Publications: Fukuda, Y., Ferrans, V. J., Schoenberger, C. I., Rennard, S. I. and Crystal, R. G.: Patterns of pulmonary structural remodeling after experimental paraquat toxicity. The morphogensis of intraalveolar fibrosis. Am. J. Pathol. 118: 452-475, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03857-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Poikilocytosis in dogs with chronic doxorubicin toxicosis.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Stephen F. Badylak, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana
John F. van Vleet, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana
Eugene H. Herman, Food and Drug Administration, Washington, D. C.
Charles E. Myers, Chief, Clinical Pharmacology Branch, NCI

COOPERATING UNITS *(if any)*

School of Veterinary Medicine, Purdue University, West Lafayette, Indiana
Clinical Pharmacology Branch, National Cancer Institute

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Poikilocytosis was observed in peripheral blood smears obtained from dogs given doxorubicin. Concurrent administration of thyroxine and ICRF-187 did not prevent the occurrence of poikilocytosis in doxorubicin-treated dogs. Administration of N-acetylcysteine with doxorubicin resuled in a mild increase in the extent of poikilocytosis compared with that observed in dogs given doxorubicin alone. The hematologic changes were not accompanied by adverse clinical signs referable to the doxorubicin-induced alterations in erythrocytes.

775

Project Descrition

Objectives: To evaluate the occurrence of poikilocytosis in dogs with chronic doxorubicin toxicosis.

Methods employed and major findings: Peripheral blood smears made during 2 studies of chemical antidotes for doxorubicin (DXR) cardiotoxicity in dogs were examined to determine the incidence of poikilocytosis. The first study showed significantly increased numbers of poikilocytes in 3 groups of5 dogs, each treated with DXR alone, DXR plus 0.5 mg/day of thyroxine, and DXR plus 2 mg/day of thyroxine. The second study showed a significant increase in poikilocytes in 4 groups of 6 dogs, each treated with DXR alone, DXR plus ICRF-187, DXR plus N-acetylcysteine (NAC), DXR plus ICRF-187 and NAC, compared to 4 control groups of 3 dogs, each treated with ICRF-187, NAC, ICRF-187 plus NAC, and physiological saline. In both studies the poikilocytes were identified as echynocytes, spiculated erythrocytes and schizocytes. Administration of thyroxin and ICRF-187 with DXR did not prevent the occurrence of poikilocytosis in DXR-treated dogs. Administration of NAC with DXR resulted in a mild decrease in the extent of poikilocytosis compared to that observed in dogs given DXR alone. The hematologic changes observed in both studies were not accompanied by adverse clinical signs referable to DXR-induced alterations in erythrocytes.

Significance: Poikilocytosis is thought to be induced by DXR as a consequence of free radical damage to the erythrocyte membrane. Studies of protection against this damage may provide useful information on the mechanisms by which DXR produces damage in other tissues. The dog provides a suitable model for these studies.

Proposed course: Project completed.

Pubications: Badylak, S. F., Van Vleet, J. F., Herman, E. H., Ferrans, V. J. and Myers, C. E.: Poikilocytosis in dogs with chronic doxorubicin toxicosis. Am. J. Vet. Res. 46: 505-508, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03858-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunocytochemical characterization of histiocytosis X cells in lung biopsies.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Yuh Fukuda, Visiting Expert, Ultrastructure Section, Pathology Branch, NHLBI
Paul Soler, INSERM, Hopital Bichat, Paris, France
Sylvie Chollet, INSERM, Hôpital Bichat, Paris, France
Claude Jacque, INSERM, Hôpital de la Salpetrière, Paris, France
Françoise Basset, INSERM, Hôpital Bichat, Paris; France

COOPERATING UNITS *(if eny)*

INSERM, Hopital Bichat and Hopital de la Salpetriere, Paris, France.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.4	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Morphologic and immunohistochemical studies were made of open lung biopsies from patients with histiocytosis X in order to detect the histiocytosis X cells that are typical of this disorder. These cells were specifically labeled by OKT6 monoclonal antibody and by antibodies against S-100 protein. The diagnostic applications of these techniques are discussed in detail.

777

Project Description

Objectives: To characterize the immunohistochemical reactivity of histiocytosis X cells in lung biopsies from patients with pulmonary histiocytosis X and related disorders.

Methods employed and major findings: Morphologic and immunohistochemical studies were made of open lung biopsies from 9 patients with histiocytosis X (HX) and 12 patients with other conditions, and of skin biopsies from patients with cutaneous sarcoidosis, Chester-Erdheim disease, and eruptive histiocytoma. The monoclonal antibody OKT6 was detected with the use of goat anti-mouse IgG labeled with fluorescein (FITC) for light microscopy, and sheep anti-mouse Fab2 fragment of IgG labeled with horseradish peroxidase (HRP) for electron microscopy. The presence of S-100 protein was revealed by an antibody prepared against bovine S-100 protein, using sheep anti-rabbit IgG labeled with FITC for light microscopy and with HRP for electron microscopy. OKT6 antibody and S-100 protein were detected simultaneously by double labeling with FITC and rhodamine. In all patients with pulmonary HX, the major cellular components (HX cells) of the granulomas showed labeling of the plasma membranes by OKT6 and of the cytoplasm by the antibody against S-100 protein. The double labeling technique demonstrated that the same cells carried both reactivities. Immunoelectron microscopy showed that the reactive cells had all the structural characteristics of Langerhans cells, including Langerhans cell granules. Cells reacting with OKT6 showed discrete internal labeling in some of the Langerhans granules, especially those in continuity with the plasma membranes. However, internal labeling of Langerhans granules was not demonstrated in preparations for the localization of S-100 protein. Control samples of sarcoid lesions and other pulmonary lesions unrelated to HX did not show any reactivity except in Langerhans cells; a skin lesion from a patient with eruptive histiocytoma contained OKT6-positive cells which lacked Langerhans granules.

Significance: The diagnosis of pulmonary histiocytosis X on the basis of histologic findings can present considerable difficulty, and the results of the present study provide a means for more accurate identification of histiocytosis X cells in lung biopsies.

Proposed course: Project completed.

Publications: Soler, P., Chollet, S., Jacque, C., Fukuda, Y., Ferrans, V. J. and Basset, F.: Immunocytochemical characterization of pulmonary histiocytosis X cells in lung biopsies. Am. J. Pathol. 118: 439-451, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03859-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*

Surgical pathology of the cardiovascular system.

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigator.) (Name, title, leboretory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Hugh A. McAllister, Jr., Chief, Department of Cardiovascular Pathology, Armed Forces Institute of Pathology, Washington, D. C.

COOPERATING UNITS *(if any)*

Department of Cardiovascular Pathology, Armed Forces Institute of Pathology, Washington, D.C.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

An extensive review was prepared of the pathology of the cardiovascular system, with emphasis on disorders that are likely to be evaluated by a surgical pathologist.

779

GPO 914-918

Project Description

Objectives: To provide a comprehensive didactic review of the pathology of the cardiovascular system to meet the needs of surgical pathologists.

Methods employed and major findings: Extensive descriptions are presented, first of the normal gross and microscopic anatomy of the cardiovascular system, and then of pathological findings in myocardium, endocardium, pericardium, valves, coronary arteries and peripheral vessels. Emphasis is placed on those conditions which are likely to come to the attention of the surgical pathologist.

Significance: This is the first review of cardiovascular pathology written especially for the surgical pathologist. This information is useful in view of the increasing number of cardiovascular pathology specimens submitted to surgical pathologists.

Proposed course: Project completed.

Publications: McAllister, H. A. Jr., and Ferrans, V. J.: Heart, pericardium and major vessels. IN: Practical Surgical Pathology, Karcioglu, A. Z. and Someren, A. (eds), The Collamore Press, D. C. Heath and Company, Lexington, MA, 1985, pp 103-155.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03860-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Comparison of doxorubicin cardiotoxicity in normotensive and hypertensive rats.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Eugene H. Herman, Food and Drug Administration, Washington, D. C.
Antoine N. El-Hage, Food and Drug Administration, Washington, D. C.
Bach Ardalan, Food and Drug Administration, Washington, D. C.

COOPERATING UNITS *(if any)*

Food and Drug Administration, Washington, D. C.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A comparison was made of the severity of chronic doxorubicin cardiotoxicity in adult male spontaneously hypertensive rats (SHR) and in genetically related normotensive Wistar-Kyoto rats. Spontaneously hypertensive rats were found to be much more sensitive than normotensive rats to the cardiotoxic effects of doxorubicin.

781

Project Description

Objectives: To compare the severity of doxorubicin-induced cardiomyopathy in normotensive and spontaneously hyptertensive rats.

Methods employed and major findings: Groups of spontaneously hypertensive (SHR) and genetically related normotensive Wistar-Kyoto (WKY) rats were given 12 weekyl injections of 0.25, 0.5 or 1.0 mg/kg of doxorubicin. When the study was concluded, mean arterial pressure was 127-161 mm Hg in doxorubicin-treated SHR compared with 74-87 mm Hg in similarly treated WKY. Lesions, consisting mainly of cytoplasmic vacuolization and myofibrillar loss, were noted in the hearts from both types of rats given the 1 mg/kg dose and were considerably more severe in SHR than in WKY (average scores of 3.8 and 2.0 on a scale of 0 to 4). Renal lesions (glomerular vacuolization and dilatation of tubules with accumulations of proteinaceous material) were of comparable severity in both types of rats at the 9 and 12 mg/kg cumulative doses; however, thety were more severe in SHR at the 6 mg cumulative dose. Moderate cardiac alterations were present in all SHR (average score 1.6) given 0.5 mg/kg of doxorubicin; at the same dose, lesions were minimal in 2 and absent in 3 WKY. In a second study, groups of rats were killed 1 week after 3, 6, 9 or 12 weekly injections of 1 mg/kg of doxorubicin. Myocardial lesions were noted initially in SHR after 6 doses and in WKY after 9 doses. Three of the 5 SHR were dead by the 12th dose.

Significance: This study shows that spontaneously hypertensive rats are much more sensitive than normotensive rats to the cardiotoxic effects of doxorubicin. Thus, they provide an unique model system in which to study various correlative aspects of this cardiotoxicity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03861-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Ultrastructure and function of the myocardium: an overview.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A review was prepared to summarize some of the cardiac structural-functional correlations that have been established during the last two decades on the basis of morphological and clinical studies. Emphasis is given to studies on cardiac hypertrophy, the cardiomyopathies and metabolic diseases.

753

Project Description

Objectives: To review recent advances in the ultrastrutural pathology of the heart.

Methods employed and major findings: This article summarizes some of the cardiac structural-functional correlations that hav been established during the last two decades on the basis of morphological and clinical studies. A review of morphologic aspects of cardiac hypertrophy is presented first, followed by reviews of structural findings in the cardiomyopathies and in selected metabolic diseases that involve the heart.

Significance: This paper presents a summary of important contributions made by ultrastructure research in the field of heart muscle diseases.

Project course: Project completed.

Publications: Ferrans, V. J. Ultrastructure and function of the myocardium: An overview of recent correlative studies. IN: Regulation of Cardiac Function. H. Abe, ed., Japan Sci. Soc. Press, Tokyo, 1985, pp 61-80.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZOI HL 03862-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Protection by ICRF-187 and its structural analogues against doaunomycin toxicity.

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Eugene H. Herman, Food and Drug Administration, Washington, D. C.
Antoine N. El-Hage, Food and Drug Administration, Washington, D. C.
Andrew M. Creighton, Imperial Cancer Research Fund, London, United Kingdom
Donald T. Witiak, College of Pharmacy, Ohio State University, Columbus, Ohio

COOPERATING UNITS *(if any)*

Food and Drug Administration, Washington, D. C.
Imperial Cancer Research Fund, London, United Kingdom
College of Pharmacy, Ohio State University, Columbus, Ohio.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Comparisons were made of the protective activity of ICRF-187 and a series of related bis-dioxopiperazine analogues against the acute toxicity produced by daunomycin (daunorubicin) in Syrian golden hamsters. The protective activity was found not to be stereospecific with respect to the l- and the d-isomers of ICRF-159, namely, ICRF-186 and ICRF-187. Protection was minimal or absent when the hamsters were pretreated with various doses of ICRF analogues in which slight changes had been made in the dioxopiperazine rings or in the central chain. Thus, very little alteration can occur in the basic structure of ICRF-187 without loss of its protective activity against anthracycline cardiotoxicity.

735

GPO 914-918

Project Description

Objectives: To compare the protective activity of ICRF-187 and a series of related bis-dioxopiperazine analogues against acute daunorubicin toxicity in Syrian golden hamsters.

Methods employed and major findings: Comparisons were made of the protective activity of ICRF-187 and a series of structurally related bis-dioxopiperazine analogues against acute toxicity produced by a single dose of 25 mg/kg of daunorubicin in Syrian golden hamsters. This dose caused a marked decrease in body weight and a mortality of 84% within 1-4 weeks. Pretreatment with ICRF-187, the d-isomer of ICRF-159, amelirated the lethal effects of daunorubicin. Over 70% of the animals given 50-200 mg of ICRF-187 before daunorubicin were alive at 8 weeks. Similar results were obtained with ICRF-186, the l-isomer of ICRF-159, indicating that the protective activity is not stereospecific. Eighteen oter analogues were also evaluated for protective activity; only bimolane, a central chain desmethyl analogue of ICRF-187 with N-morpholinomethyl susbtituents in each dioxopiperazine ring, was as effective as ICRF-187 in reducing the mortality of daunorubicin. The role of the N-morpholinomethyl groups in the biological activity of bimolane needs further study, since ICRF-154, a similar compound without these substituents, exerted only minimal protective activity. Protection against daunorubicin lethality was minimal or absent when hamsters were pretreated with various doses of ICRF analogues in which slight changes had been made in the dioxopiperazine rings (ICRF-158, ICRF-198) or in the central chain (ICRF-161, ICRF-192, ICRF-193, ICRF0-197, ICRF-198 and ICRF-202). Similarly, animals pretreated with a number of conformationally constrained cyclopropane analogues of bis-dioxopiperazine compounds before receiving daunorubicin died at the same rates as those given only daunorubicin.

Significance: Previous studies from this unit have established the effectiveness of ICRF-187 in preventing the cardiotoxicity produced by the administration of doxorubicin and daunorubicin, two highly potent antineoplastic agents. The present study of analogues of ICRF-187 indicates that little alteration can occur in the structure of this compound without loss of its protective activity.

Project course: Project completed.

Publications: Herman, E. H., El-Hage, A. N., Creighton, A. M., Witiak, D. and Ferrans, V. J.: Comparison of the protective effect of ICRF-187 and structurally related analogues against acute daunorubicin toxicity in Syrian golden hamsters. Res. Commun. Chem. Pathol. Pharmacol. 48: 39-55, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03863-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA synthesis in rat atrial myocytes as a response to left ventricular infarction.

PRINCIPAL INVESTIGATOR *(List other profassional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI

John O. Oberpriller, Guest Scientist, Ultrastructure Section, Pathology Branch, NHLBI

Raymond J. Carroll, Department of Statistics, University of North Carolina, Chapel Hill, N.C.

COOPERATING UNITS *(if any)*

Department of Statistics, University of North Carolina

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Autoradiographic studies were made on enzymatically dissociated atrial myocytes to examine the DNA synthetic response of atrial myocardium to left ventricular infarction. These studies showed that increased DNA synthesis, manifested as increased labeling of myocytes with tritiated thymidine, occurs in atrial myocardium as a response to left ventricular infarction.

737

Project Description

Objectives: To study the DNA synthetic response of atrial myocytes to ventricular infarction.

Methods used and major findings: An autoradiographic study was performed on enzymatically dissociated atrial muscle cells to examine the DNA synthetic response of atria to left ventricular infarction. DNA synthesis was studied in left and right atrial myocytes and nonmyocyte cells of: 1) young Sprague-Dawley rats 11 days after ligation of the left coronary artery; 2) rats subjected to a shm surgical procedure without coronary artery ligation, and 3) unoperated rats. Each aniaml received a series of 10 injections of tritiated thymidine, at 12 hour intervals, beginning on the 5th postoperative day; cells were isolated 36 hours after the last injection. In infarcted animals, 37.1% of the left atrial myocytes were labeled and binucleated, and 6.5% were labeled and mononucleated; 13% of right atrial myocytes were labeled and binucleated, while 12.7% were labeled and mononucleated. For both the left and right atria, the incidence of tritiated thymidine label in myocytes of the sham-operated group was similar to that of unoperated controls, indicating that the surgical procedure did not stimulate DNA synthesis in atrial myocytes. In both left and right atria of the infarcted group, non-muscle cells were labeled to a greater extent (49.9 and 47.1%) than in the sham-operated group (22 and 20.8%); these in turn showed labeling to a greater extent than did the unoperated control group (10.9 and 11.6%), indicating that DNA synthesis was stimulated in non-myocytes of the atria by the sham operation and was further stimulated by the experimental infarction. A low level of labeling occurred in myocytes and non-myocytes in unoperated animals, indicating that a small amount of DNA synthesis was occurring in atrial tissue in the young, growing rats used in this study.

Significance: The present study shows thatatrial myocardium responds to left ventricular infarction by undergoing a certain amount of DNA synthesis. Such a snthesis leads to a combination of binucleation and increase in ploidy. It remains uncertain whether these changes can progress to complete cell division.

Porject course: Project completed.

Publications: Oberpriller, J. O., Ferrans, V. J. and Carroll, R. J.: DNA synthesis in rat atrial myocytes as a response to left ventricular infarction. An autoradiographic study of enzymatically dissociated myocytes. J. Mol. Cell. Cardiol. 16: 1119-1126, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03864-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Anorexia Nervosa and Sudden Death

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jeffrey M. Isner - Tufts-New England Medical Center, Boston
William C. Roberts - Chief, Pathology Branch - NHI
Steven B. Heymsfield - Emory University School of Medicine
Joel Yager - Neuropsychiatric Institute, University of California

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NIH/NHLBI/Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Necropsy findings and electrocardiograms from three woman with anorexia nervosa were reviewed. Necropsy examination failed to establish an anatomic cause of death. Electrocardiograms recorded 7 days or less before death showed various degrees of Q-T interval prolongations: Q-T intervals corrected for heart rate measured 0.61 s, 0.47 s, and 0.46 s, respectively. Terminal ventricular tachyarrhythmias were documented in two patients, including torsade de pointes in one. The necropsy and clinical findings in these three cases provide evidence that sudden death in anorexia nervosa, like sudden death in liquid-protein dieting, may result from ventricular tachyarrhythmias related to Q-T interval prolongation. For such patients, electrocardiographic monitoring should be routine.

PHS 6040 (Rev. 1/84) GPO 904-917

Gross Description:

Necropsy findings and electrocardiograms from three woman with anorexia nervosa were reviewed. Necropsy examination failed to establish an anatomic cause of death. Electrocardiograms recorded 7 days or less before death showed various degrees of Q-T interval prolongations: Q-T intervals corrected for heart rate measured 0.61 s, 0.47 s, and 0.46 s, respectively. Terminal ventricular tachyarrhythmias were documented in two patients, including torsade de pointes in one. The necropsy and clinical findings in these three cases provide evidence that sudden death in anorexia nervosa, like sudden death in liquid-protein dieting, may result from ventricular tachyarrhythmias related to Q-T interval prolongation. For such patients, electrocardiographic monitoring should be routine.

Publication:

Isner JM, Roberts WC, Heymsfield SB, Yager J. Anorexia Nervosa and Sudden Death. Ann Int Med 1985. 102:49-52.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03865-01 PA

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The King Of Hearts: Analysis of 23 Patients with Hearts Weighing 1,000 Grams or More

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

William C. Roberts - Chief, Pathology Branch, NHLBI

Michael J. Podolak - Student - Duke University Medical School

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NIH/NHLBI - Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Certain clinical and morphologic features are described in 23 patients in whom the heart at necropsy weighed at least 1,000 g (mean 1,106). The heart weight to body weight ratio ranged from 1.2 to 2.7 (normal 0.40). The 23 patients were derived from examination of the hearts of 7,671 patients with various cardiovascular disorders over a 25-year period. The massive cardiomegaly was the result of aortic regurgitation in 14 patients (61%): isolated in 8, associated with mitral regurgitation in 4, and with ventricular septal defect in 2. Three others (13%) had combined aortic valve stenosis and aortic regurgitation and 1 patient (4%) had mitral stenosis and regurgitation and mild aortic stenosis. Four patients (17%) had hypertrophic cardiomyopathy, and 1 patient (4%) had ventricular septal defect with mitral stenosis. They were 20 to 64 years old (mean 442) and 21 (91%) were men. Four patients at necropsy had 1 or more major coronary arteries narrowed more than 75% in cross-sectional area by atherosclerotic plaques, and only 4 patients had grossly visible left ventricular (LV) scars, 2 of whom had insignificant coronary narrowing. Examination of electrocardiograms in 17 of the 23 patients disclosed that Sokolow-Lyon criteria for LV hypertrophy was achieved in only 12 patients (71%) and Romhilt-Holt QRS voltage criteria faired even worse. Total 12-lead QRS voltage was more than 175 mm (10 mm = 1mV) in 16 patients (94%) and it was more than 250 mm in 13 patients (76%). Total 12-lead QRS voltage in 17 patients ranged from 140 to 601 mm (mean 323). Measurement of the sum in diagnosing LV hypertrophy by electrocardiogram.

791

Gross Description:

Certain clinical and morphologic features are described in 23 patients in whom the heart at necropsy weighed at least 1,000 g (mean 1,106). The heart weight to body weight ratio ranged from 1.2 to 2.7 (normal 0.40). The 23 patients were derived from examination of the hearts of 7,671 patients with various cardiovascular disorders over a 25-year period. The massive cardiomegaly was the result of aortic regurgitation in 14 patients (61%): isolated in 8, associated with mitral regurgitation in 4, and with ventricular septal defect in 2. Three others (13%) had combined aortic valve stenosis and aortic regurgitation and 1 patient (4%) had mitral stenosis and regurgitation and mild aortic stenosis. Four patients (17%) had hypertrophic cardiomyopathy, and 1 patient (4%) had ventricular septal defect with mitral stenosis. They were 20 to 64 years old (mean 442) and 21 (91%) were men. Four patients at necropsy had 1 or more major coronary arteries narrowed more than 75% in cross-sectional area by atherosclerotic plaques, and only 4 patients had grossly visible left ventricular (LV) scars, 2 of whom had insignificant coronary narrowing. Examination of electrocardiograms in 17 of the 23 patients disclosed that Sokolow-Lyon criteria for LV hypertrophy was achieved in only 12 patients (71%) and Romhilt-Holt QRS voltage criteria faired even worse. Total 12-lead QRS voltage was more than 175 mm (10 mm = 1mV) in 16 patients (94%) and it was more than 250 mm in 13 patients (76%). Total 12-lead QRS voltage in 17 patients ranged from 140 to 601 mm (mean 323). Measurement of the sum in diagnosing LV hypertrophy by electrocardiogram.

Publication:

Roberts, WC, Podolak MJ. The King of Hearts: Analysis of 23 Patients with Hearts Weighing 1,000 Grams or More. Am J Cardiol 1985; 55:485-494.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03866-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Detailed Anatomy of the Normally Functioning Aortic Valve in Hearts of Normal

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Marc A. Silver - Rush-Presbyterian -St.Luke's Medical Center - Chicago

William C. Roberts - Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NIH/NHLBI - Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The area, weight and 4 linear variables were measured in each aortic valve cusp in 100 necropsy patients with normally functioning aortic valves, and the voluem of each sinus of Valsalva and the aortic area at the sinotubular junction were determined in the same patients. The sums of the aortic valve cuspal areas, cuspal weights and sinus of Valsalva volumes increased with age ($p < 0.001$) and with heart weight ($p < 0.001$). All 3 variables (cuspal area, cuspal weight and sinus of Valsalva volume) also increased with age and heart weight relative to each other. The luminal area of aorta at the sinotubular junction also increased with age and heart weight and it also increased as the sum of the aortic valve cuspal areas and weights and sinus of Valsalva volumes increased. In only 16% of the 100 patients were the 3 aortic valve cusps of similar size (less than 5% difference in area between cusps); in 51%, 1 cusp was of different size than the other 2, and in 33% of patients all 3 cusps were of different sizes.

PHS 6040 (Rev 1/84)

GPO 904-917

Gross Description:

The area, weight and 4 linear variables were measured in each aortic valve cusp in 100 necropsy patients with normally functioning aortic valves, and the voluem of each sinus of Valsalva and the aortic area at the sinotubular junction were determined in the same patients. The sums of the aortic valve cuspal areas, cuspal weights and sinus of Valsalva volumes increased with age ($p < 0.001$) and with heart weight ($p < 0.001$). All 3 variables (cuspal area, cuspal weight and sinus of Valsalva volume) also increased with age and heart weight relative to each other. The luminal area of aorta at the sinotubular junction also increased with age and heart weight and it also increased as the sum of the aortic valve cuspal areas and weights and sinus of Valsalva volumes increased. In only 16% of the 100 patients were the 3 aortic valve cusps of similar size (less than 5% difference in area between cusps); in 51%, 1 cusp was of different size than the other 2, and in 33% of patients all 3 cusps were of different sizes.

Publication:

Silver MA, Roberts WC. Detailed Anatomy of the Normally Functioning Aortic Valve in Hearts of Normal and Increased Weight. Am J Cardiol 1985; 55:454-461.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 HL 03867-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Fatal Water Intoxication, Schizophrenia and Diuretic Therapy for Systemic Hypertensi

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Stewart Levine, MD	Cardiology Fellow, Georgetown
Bruce M. McMannus, MD, Ph.D.	Staff Fellow, Pathology Branch, NHLBI
Brian D. Blackbourne, MD	Staff, DCMEO, DC
William C. Roberts, MD	Chief, Pathology Branch

COOPERATING UNITS *(if any)*

LAB/BRANCH

NHLBI/Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH/NHLBI - Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c). Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Clinical and morphologic findings are described on a 37-year old hypertensive man with chronic schizophrenia who had 2 well-documented episodes of water intoxication. The use of diuretics for control of systemic hypertension in the setting of chronic schizophrenia appears ill-advised.

PHS 6040 (Rev 1/84)

GPO 904-917

Gross Description:

Clinical and morphologic findings are described on a 37-year old hypertensive man with chronic schizophrenia who had 2 well-documented episodes of water intoxication. The use of diuretics for control of systemic hypertension in the setting of chronic schizophrenia appears ill-advised.

Publication:

Levine, S, McManus, BM, Blackbourne, BD, Roberts WC. Fatal water intoxication, schizophrenia and diuretic therapy for systemic hypertension. Submitted for publication in the American Journal of Medicine.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03868-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Electrocardiographic Observations in Clinically Isolated, Pure, Chronic, Severe Aortic Regurgitation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

William C. Roberts - Chief, Pathology Branch, NHLBI

Paul J. Day - Student, Saint Mary's College, Maryland

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NIH/NHLBI - Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Certain electrocardiographic findings are described in 30 necropsy patients with clinically isolated pure, chronic, severe aortic regurgitation. They were 19 to 65 years old (mean 45). The hearts of the 22 men ranged in weight from 430 to 1,110 g (mean 717) and of the 8 women, from 375 to 950 g (mean 638). Four had grossly visible left ventricular (LV) scars. All but 1 patient was in sinus rhythm. The PR interval was >0.20 second in 8 patients (28%) and the QRS duration was $\geq$ 0.12 second in 6 patients (20%). Only 5 patients (17%) had 1 or more ventricular premature complexes recorded on the resting electrocardiogram analyzed. The mean QRS amplitude for each of the 12 leads average 23 mm. The highest mean QRS voltage occurred in leads V_2 and V_3 (each 38 mm), and the lowest in lead aVR (11 mm). The mean QRS voltage in V_5 was higher than V_6 (33 vs 28 mm) and in 22 patients (73%) the QRS voltage in V_5 was higher than in V_6. The sum of the S wave in V_1 plus the larger of the R wave in V_5 or V_6 (Sokolow-Lyon index) averaged 51 mm and in only 22 patients (73%) was it >35 mm.

797

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Publication:

Roberts WC, Day PJ. Electrocardiographyic Observations in Clinically Isolated Pure, Chronic, Severe Aortic Regurgitation: Analysis of 30 Necropsy Patients Aged 19 to 65 Years. Am J Cardiol 1985; 55:431-438.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03869-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Remnant Saphenous Veins After Aortocoronary Bypass Grafting: Analysis of 3,3394

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Bruce F. Waller - Department of Pathology, University Hospital, Indianapolis

William C. Roberts - Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NIH/NHLBI - Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

To assess the status of a saphenous vein (SV) excised for coronary artery bypass grafting (CABG), 3,394 cm of remnant SV from 402 patients who underwent CABG were examined. The SV remnants were 0.5 to 52 cm long (mean 8.4). They were sectioned into 5-mm-long segments, and the resulting 6,788 five-millimeter segments were examined histologically: 5,896 (87%) were narrowed 0 to 25% in cross-sectional area by fibrous tissue; 853 (12%) were narrowed 26 to 50%; 23 (0.6%), 51 to 75%, and 16 (0.4%) segments were narrowed 76 to 100%. Of the 16 segments severely narrowed, 7 (44%) were nearly totally occluded by fibrous tissue. In 17 patients who died within 24 hours of CABG, similar degrees of luminal narrowing were observed in remnant segments and in utilized segments of SV. Thus, significant preexisting luminal narrowing of SV used for CABG is infrequent. The intimal fibrous thickening is variable within the same vein when each 5-mm-long segment is analyzed; it is variable from 1 vein to another in the same patient, and it varies among patients.

799

Gross Description:

To assess the status of a saphenous vein (SV) excised for coronary artery bypass grafting (CABG), 3,394 cm of remnant SV from 402 patients who underwent CABG were examined. The SV remnants were 0.5 to 52 cm long (mean 8.4). They were sectioned into 5-mm-long segments, and the resulting 6,788 five-millimeter segments were examined histologically: 5,896 (87%) were narrowed 0 to 25% in cross-sectional area by fibrous tissue; 853 (12%) were narrowed 26 to 50%; 23 (0.6%), 51 to 75%, and 16 (0.4%) segments were narrowed 76 to 100%. Of the 16 segments severely narrowed, 7 (44%) were nearly totally occluded by fibrous tissue. In 17 patients who died within 24 hours of CABG, similar degrees of luminal narrowing were observed in remnant segments and in utilized segments of SV. Thus, significant preexisting luminal narrowing of SV used for CABG is infrequent. The intimal fibrous thickening is variable within the same vein when each 5-mm-long segment is analyzed; it is variable from 1 vein to another in the same patient, and it varies among patients.

Publication:

Waller BF, Roberts WC. Remnant Saphenous Veins after Aortocoronary Bypass Grafting: Analysis of 3,394 Centimeters of Unused Vein from 402 Patients. Am J Cardiol 1985; 55:65-71.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZOI HL 03870-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Severe Atherosclerotic Coronary Arterial Narrowing and Chronic Congestive Heart

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Elizabeth M. Ross - Senior Staff Fellow - Pathology Branch, NHLBI

William C. Roberts - Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NIH/NHLBI/Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We studied at necropsy 18 patients (aged 38 to 73 years [mean 58]; 16 [89%] men) with chronic congestive heart failure (CHF) of >3 months duration, >75% cross-sectional area (XSA) narrowing of ≥1 of the 4 major epicardial coronary arteries by atherosclerotic plaque, and no left ventricular (LV) fibrosis or necrosis. Because grossly visible myocardial lesions were absent, the severe chronic CHF in these 18 patients cannot reasonably be attributed to CAD. It is most reasonable to believe that this group of patients had idiopathic dilated cardiomyopathy and the CAD was coincidental.

801

Gross Description:

We studied at necropsy 18 patients (aged 38 to 73 years [mean 58]; 16 [89%] men) with chronic congestive heart failure (CHF) of >3 months duration, >75% cross-sectional area (XSA) narrowing of ≥1 of the 4 major epicardial coronary arteries by atherosclerotic plaque, and no left ventricular (LV) fibrosis or necrosis. Because grossly visible myocardial lesions were absent, the severe chronic CHF in these 18 patients cannot reasonably be attributed to CAD. It is most reasonable to believe that this group of patients had idiopathic dilated cardiomyopathy and the CAD was coincidental.

Publication:

Ross, EM, Roberts, WC. Severe Atherosclerotic Coronary Arterial Narrowing and Chronic Congestive Heart Failure Without Myocardial Infarction: Analysis of 18 Patients Studied at Necropsy. Accepted for publication in the Am. J. Cardiol.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03871-01 PA

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Severe Atherosclerotic Coronary Artery Disease, Healed Myocardial Infarction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Elizabeth M. Ross, - Senior Staff Fellow, Pathology Branch, NHLBI

William C. Roberts, Chief, Pathology Branch

COOPERATING UNITS *(if any)*

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NHLBI/NIH/Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Certain observations are described in 81 necropsy patients (aged 29-91 years [mean 62]; 77 [95%] men) with severe congestive heart failure (CHF) of >3 months duration, left ventricular (LV) transmural scar, and >75% cross-sectional area (XSA) narrowing by atherosclerotic plaque of 1 or more of the 4 mjor epicardial coronary arteries. The duration of symptoms from initial onset of acute myocardial infarction (59 patients) or CHF (18 patients) or angina pectoris (2 patients) to death ranged from 0.5 to 18 years (mean 7.1) (2 unknown). Angina pectoris occurred sometime, however, (59%); sudden (arrhythmia) in 16 (20%); acute myocardial infarction in 11 (14%), and emboli in 6 (7%). The heart weight ranged from 410 to 800 g (mean 585). Left and/or right ventricular thrombi occurred in 37 patients (46%), only 4 (10%) of whom had systemic emboli; of the 44 patients without intracardiac thrombi, none had emboli. The severity of coronary narrowing was variable. In 24 patients (30%) only 1 artery was narrowed >75% in XSA; in 22 patients (27%), 2 arteries were so narrowed, in 32 patients (39%), 3 arteries, and in 3 patients (4%), 4 arteries were so narrowed. The size of the LV scar also varied. Of the 81 patients, 58 (72%) had large scars (involving >40% of the LV wall); 10 (12%) had moderate sized scars (6-40% of the LV wall); and 13 (16%) had small scars (≥5% of the LV wall). The size of the LV scar correlated with a history of habitual alcoholism: of the 16 habitual alcoholics, 6 (38%) had small and 8 (50%) had large LV scars; of the 65 non-alcoholics, 7 (11%) had small and 50 (77%) had large LV scars ($p < 0.05$). The chronic CHF in the 68 patients with either moderate or large-sized LV scars is readily attributed to the LV damage; in the 13 patients with small LV scars, however, the chronic CHF more reasonably may be attributed to another factor, e.g. alcoholism, despite coronary artery narrowing similar in severity to that in the patients with large LV scars.

803

Project Description:

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Publication:

Ross EM, and Roberts WC. Severe Atherosclerotic Coronary Artery Disease, Healed Myocardial Infarction and Chronic Congestive Heart Failure: Analysis of 81 Patients Studied at Necropsy. Accepted for publication in AJC.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03872-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Relation of size of Transmural Acute Myocardial Infarction to Mode of Death

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jeffrey E. Saffitz, MD, PHD - Department of Pathology - Washington University School of Medicine - St. Louis, MO

Rurik C. Fredrickson - Pathology Branch, NHLBI

Willicam C. Roberts, MD Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION

NIH/NHLBI/Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Hearts of 78 necropsy patients with transmural acute myocardial infarction were studied to correlate the mode of death, the interval between onset of infarction and death, and the presence or absence of coronary thrombus with the extent of the infarct. Infarct size was assessed quantitatively as a percent of total left ventricular mass. Death was caused by cardiogenic shock in 16 patients (21%), arrhythmia in 31 patients (40%), and cardiac rupture in 24 patients (13%). The mean interval between the onset of acute myocardial infarction and death was 12 ± 13 days. Infarct size averaged 23 ± 14% of left ventricular mass. Patients who died in cardiogenic shock had the largest infarcts (37 ± 11%) and those dying of cardiac rupture had the smallest infarcts (15 ± 9%) and the shortest interval between onset of infarction and death (7 ± 8 days). Coronary thrombi were present in 58 patients (74%). When present, thrombus was observed in the coronary artery which had supplied the infarct area and was superimposed on advanced atherosclerotic plaque but there was no apparent relation between the extent of luminal obstruction by thrombus and infarct size. The absence of coronary thrombus at necropsy was associated with either small infarcts or prolonged survival following infarction.

Gross Description:

Hearts of 78 necropsy patients with transmural acute myocardial infarction were studied to correlate the mode of death, the interval between onset of infarction and death, and the presence or absence of coronary thrombus with the extent of the infarct. Infarct size was assessed quantitatively as a percent of total left ventricular mass. Death was caused by cardiogenic shock in 16 patients (21%), arrhythmia in 31 patients (40%), and cardiac rupture in 24 patients (13%). The mean interval between the onset of acute myocardial infarction and death was 12 + 13 days. Infarct size averaged 23 + 14% of left ventricular mass. Patients who died in cardiogenic shock had the largest infarcts (37 + 11%) and those dying of cardiac rupture had the smallest infarcts (15 + 9%) and the shortest interval between onset of infarction and death (7 + 8 days). Coronary thrombi were present in 58 patients (74%). When present, thrombus was observed in the coronary artery which had supplied the infarct area and was superimposed on advanced atherosclerotic plaque but there was no apparent relation between the extent of luminal obstruction by thrombus and infarct size. The absence of coronary thrombus at necropsy was associated with either small infarcts or prolonged survival following infarction.

Publication:

Saffitz, JE, Fredrickson, RC, Roberts, WC. Relation of Size of Transmural Acute Myocardial Infarction to Mode of Death, Interval Between Infarction and Death, and Frequency of Coronary Arterial Thrombus.
Submitted to AJC.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 03873-01 PA

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Left Main Coronary Artery Originating from the Right Sinus of Valsalva and Coursing

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Charles W. Barth - Senior Staff Fellow - Pathology Branch, NHLBI

William C. Roberts - Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH: Pathology Branch/NHLBI

SECTION:

INSTITUTE AND LOCATION: NIH/NHLBI - Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Findings are described in 5 patients who at necropsy were found to have origin of the left main coronary artery from the right sinus of Valsalva and coursing of the anomalously arising artery between aorta and pulmonary trunk to reach the left side of the heart. Three (all boys) of the 5 patients died suddenly at 13, 14 and 19 years, respectively. 2 of them had had 1 or more episodes of syncope and the third had an abnormal electrocardiogram. The fourth patient, a 64-year-old woman, died of chronic congestive heart failure 1 year after an acute myocardial infarction. She had insignificant coronary atherosclerosis. The fifth patient, an 81-year-old man, died of chronic alcoholism, having been free of symptoms of cardiac dysfunction during life. Additionally, clinical and necropsy findings are summarized in 38 previously report necropsy patients with the coronary anomaly. Of the 38 (34 males [89%], 23 (61%) died suddenly in the first 2 decades of life; death in 6 (16%) others appears to have been related to coronary atherosclerosis and 9 patients (24%) died from non-coronary causes. Thus, this anomaly is life threatening. Why it frequently causes fatal cardiac arrest in some young individuals and allows a normal life span in others remains unclear.

507

Gross Description:

Findings are described in 5 patients who at necropsy were found to have origin of the left main coronary artery from the right sinus of Valsalva and coursing of the anomalously arising artery between aorta and pulmonary trunk to reach the left side of the heart. Three (all boys) of the 5 patients died suddenly at 13, 14 and 19 years, respectively. 2 of them had had 1 or more episodes of syncope and the third had an abnormal electrocardiogram. The fourth patient, a 64-year-old woman, died of chronic congestive heart failure 1 year after an acute myocardial infarction. She had insignificant coronary atherosclerosis. The fifth patient, an 81-year-old man, died of chronic alcoholism, having been free of symptoms of cardiac dysfunction during life. Additionally, clinical and necropsy findings are summarized in 38 previously report necropsy patients with the coronary anomaly. Of the 38 (34 males [89%], 23 (61%) died suddenly in the first 2 decades of life; death in 6 (16%) others appears to have been related to coronary atherosclerosis and 9 patients (24%) died from non-coronary causes. Thus, this anomaly is life threatening. Why it frequently causes fatal cardiac arrest in some young individuals and allows a normal life span in others remains unclear.

Publication:

Barth, CW, Roberts WC. Left Main Coronary Artery Originating from the the Right Sinus of Valsalva and Coursing Between Aorta and Pulmonary Trunk. Accepted for publication in the Journal of the American College of Cardiology.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03874-01 PA

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Valve Excision only versus valve excision plus replacement for active infective

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Deborah J. Barbour, MD — Senior Staff Fellow, Patholoby Branch, NHLBI

William C. Roberts, MD — Chief, Pathology Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Patholoy Branch/NHLBI

SECTION

INSTITUTE AND LOCATION

NIH/NHLBI/ - Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
416	416	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use stanoard unreduced type. Do not exceed the spece provided.)*

This paper examines the question of the preferred surgical treatment for active infective endocarditis involving the tricuspid valve amont intravenous drug users. Two patients are presented: 1) underwent valve excision with concomitant valve bioprosthesis placement with rapid reinfection of the bioprosthesis necessitating its explantation. Redevelopment of congestive heart failure required placement of a second bioprosthesis with good results. The second patient underwent excision only with an asymptomatic state thereafter until the time of his death from overdose 11 months later. The literature is reviewed on this therapeutic dilemma with the conclusion being that excision of the infected tircuspid valve alone is preferable to excision with concurrent prosthetic replacement with the realization that congestive heart failure may make eventual valve prosthesis placement necessary in a subset of patients.

Gross Description:

This paper examines the question of the preferred surgical treatment for active infective endocarditis involving the tricuspid valve amont intravenous drug users. Two patients are presented: 1) underwent valve excision with concomitant valve bioprosthesis placement with rapid reinfection of the bioprosthesis necessitating its explantation. Redevelopment of congestive heart failure required placement of a second bioprosthesis with good results. The second patient underwent excision only with an asymptomatic state thereafter until the time of his death from overdose 11 months later. The literature is reviewed on this therapeutic dilemma with the conclusion being that excision of the infected tircuspid valve alone is preferable to excision with concurrent prosthetic replacement with the realization that congestive heart failure may make eventual valve prosthesis placement necessary in a subset of patients.

Publication:

Barbour, DJ, Roberts, WC. Valve excision only versus valve excision plus replacement for active infective endocarditis involving the tricuspid valve. To be published.

ANNUAL REPORT OF THE SURGERY BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1984 through September 30, 1985

The research performed by the Surgery Branch is directed toward problems in clinical cardiac surgery which require multifacited collaboration in basic and applied fields. The areas addressed include: 1) prosthetic heart valves; 2) ischemic heart diseases; 3) myocardial preservation; 4) cardiomyopathy and 5) surgical assessment of new ultrasonic technologies. The individual research projects reflect a broad but coherent theme of seeking answers to difficult and longstanding problems in surgical palliation of heart disease.

Prosthetic Heart Valves

All FDA approved prosthetic heart valves provide good short-term (0-3 yr) palliation. Of significant concern is the long-term relentless complications of thromboemboli, anticoagulation, material wear, fatigue, or calcification (bioprostheses). Additional clinical problems involve the lack of an adequate prosthesis for replacement of the tricuspid valve, valve replacement in children, and the high incidence of low cardiac output death after mitral valve replacement for chronic severe mitral regurgitation especially when associated with coronary artery disease. In vitro studies have begun using new testing devices which characterize prototype valves in terms of forward and backward power loss, regurgitant fraction during the closing and closed phases of the cycle and comparative hydraulic efficiencies. Durability studies are conducted using two or six valve systems in which valves are simultaneously cycled at rates of 1,000-1,200 per minute. The data, to date, for an investigational pericardial valve after short-term in vivo implantation demonstrate asynchronous opening and closing of nonstiffened leaflets. These correlate to in vitro data current in vivo data which show that bioprostheses as currently manufacturered do not have uniform functional characteristics. These data will be important in future regulatory considerations. Jones and Ferrans continue their studies of the mechanism(s) of calcification of bioprostheses. More then 500 mitral and tricuspid valve replacements using bioprostheses in juvenile sheep have been performed by Jones in the past four years. The physiologic, morphologic, ultrasonic assessments and electron microscopic studies were recently reported. The data demonstrate that juvenile sheep provide an accelerated model of the human pathologic response to bioprosthesis and is similar to that seen in children. Further, bioprostheses are mildly to moderately obstructive to forward flow in the sized used (25 mm) and provide nonuniform flow fields. Calcification is severe at 20 weeks with the present clinical valves and begins in the areas of highest stress. The role of mineralization mitigation procedures has been studied. The use of surfactants, toludine blue, non-calcium and phosphorous bearing solutions and new buffers has been shown to be efficacious. Quantification of calcium content in the leaflets of the clinical bioprostheses ranged from 83-190 mg/g of leaflet tissue. Non-implanted valves had an average value of 0.9 ± 0.1 mg/g. After porcine aortic valves were specially processed by two methods, the calcium content was decreased approximately equally from 77 to 9 mg/g, however, when the same processes were applied to bovine pericardial valves no significant reduction in calcium content was found. Five types of new synthetic tri-leaflet valves have been tested, none of which had improved in vivo longivity compared to valves approved for clinical use. Polyurethane and polytetrafluorethylene valves developed

calcification and tears. Co-polymer valves had alteration of leaflet geometry and developed central regurgitation. Two related projects concerning the problem of chronic mitral regurgitation have been initiated. The first is a laboratory study in which an animal analog of chronic mitral regurgitation has been developed. Pressure, flow and LV dimensional data are obtained before and after valve replacement with and without excision of the mitral valvular apparatus. A clinical study has been initiated in which selected patients receive mitral valve replacement without valve excision. Hemodynamic and echocardiographic studies have shown no incidence of low cardiac output syndrome and minimal changes in left ventricular dimensions. The hypothesis tested in both studies is that the mitral valve apparatus, in its entirety, supports the left ventricle and prevents left ventricular dilation after valve replacement in dilated left ventricles with chronic mitral insufficiency.

Ischemic Heart Disease

Three new projects have been initiated in the past year in collaboration with the Cardiology Branch, NHLBI and the Physics section of BEIB. These are laser-angioscopy, laser drilling of the ischemic myocardium and flow augmentation by implantation of internal mammary arteries. The laser-angioscopy project has met the following milestones: 1) completed evaluation of various fiberoptic devices suitable for angioscopy; 2) coupled the angioscope to a mini TV camera; 3) fabricated and tested infusion sheaths; 4) completed tissue-laser interaction studies using CO_2, Nd-Yag, argon, xenon fluoride and krypton chloride; 5) assessed optic wave guides for laser energy efficiencies, and 6) developed rabbit and swine atherosclerosis models. The angioscopic studies have used dog, swine, rabbit, sheep, and postmortem human species. All major coronary vessels were visualized in the larger species with the 1.5 mm diameter device. Clarity was excellent and resolution was good. Additionally, the carotid artery within the skull and anatomy of the femoral arterial system to the ankle level have been visualized. Clinical trials have begun to visualize the native coronary arteries and bypass grafts during aortic and coronary artery bypass operations. The laser-tissue thermal diffusion and histologic interaction studies have defined three possible energy sources for atheroma ablation: carbon dioxide, ultraviolet and ND-Yag. CO_2 could be used if the pulse durations were very short and the energy levels very high. Some charring from thermal effects was present with the best conditions. The ND-Yag systems require the wave-lengths to be in the range of 250-300 NM which requires a custom control system. The most readily available and most efficacious was an excimer system which will use either xenon chloride or krypton fluoride. A computer controlled system has been purchased. The optical wave guide system is in hand and appears to have sufficient efficiency to prevent wave guide damage. Two animal species have been given atherolosclerosis; rabbit and swine. Each is fed a high-cholesterol high-fat diet. The endothelium of the porcine carotid and femoral arteries was denuded by a balloon catheter which resulted in localized severe atherosclerotic obstructions three to six months later. The excimer-ablation studies will use these atherosclerotic preparations as an initial test of safety and efficacy of laser angioplasty.

Both CO_2 and excimer lasers will be used for myocardial drilling studies. Carbon dioxide or excimer lasers will be used to obtain larger perforations of 300-1,000 um from the epicardium to the mid-myocardium. Together with angiogenesis factors, endothelial seeding and application of overlay vascularized pedicles of omentum and/or spleen it is proposed that new

vasculature can be developed to the ischemic myocardium. Excimer drilling will create smaller perforations (50-300 um) to penetrate the full thickness of the left ventricular wall. The external orifices will seal or be sutured. The hypothesis is that the systolic pressure in the left ventricle will cause the small perforations to remain patent and form loci for extensive collateral vessel formation.

The internal mammary artery (IMA) implantation study has used fifty dogs to date. Dogs have had single and double implants with the application of one or two ameroid constrictors to the left anterior descending and circumflex coronary arteries. Flow augmentation has been assessed by left ventricular power functions, wall motion analyses, direct measurement of the IMA flow microsphere determination of blood flow distribution, and postmortem injection studies. The data demonstrate that the IMA patency rates were 100% with an average flow of 8 ml/min when implanted into ischemic myocardium. Microsphere studies showed that the implant supplied an additional 10-20% of myocardial segment flow (0.15 ml/gm) at rest which increased 33% with catecholamine stimulation. Additional augmentation methods will be added to this procedure which include splenic overlay with autologous endothelial seeding of the perforated surfaces. A clinical study has been initiated based on the concept that coronary vasomotor tone is dynamic in the immediate post bypass grafting interval. A thermal dilution catheter is placed in the coronary sinus under fluoroscopic guidance and advanced to the great anterior vein. Coronary sinus flow, regional oxygen consumption and various metabolic variables are measured intermittantly. These studies may provide clues to the mechanisms of perioperative infarction in patients with coronary artery disease.

Myocardial Preservation

Essential to successful surgical palliation of heart diseases of all types is the capability of protecting the heart during long intervals (2-5 hours) of global ischemia. The conventional use of hypothermia and cardioplegic solutions which cause diastolic arrest by ionic depolarization are inadequate in hypertrophied hearts made ischemic for long intervals. Various additives have been analyzed using isolated heart preparations from spontaneously hypertensive rats with right and left ventricular hypertrophy. Phentolamine, amiodarone, and prostaglandin (PBx) have been studied at normothermia (37-38^{o}C) and hypothermia (20-25^{o}C). Work function studies, high energy phosphate concentrations and pathologic studies have shown recovery of function to 70% in LD-100 preparations which were hearts made ischemic for 40 min at 38^{o}C. Extensive dose response studies were conducted for each of the three compounds. The greatest recovery of function was achieved with doses of 10 mg, 0.25 mg and 100 mg of phentolamine, amiodarone and prostaglandin PBx, respectively. A newly developed miniaturized dual pH and temperature transducer which measures interstitial conditions has been used to correlate pH values to functional recovery of the left ventricle after global ischemia and reperfusion in sheep. Myocardial temperature, infusion pressure and the temperature and frequency of infusions have been studied. The data demonstrate that a terminal ischemic pH of 6.8 correlates with 50-60% recovery in all species under all conditions. High flows and pressures were found to be beneficial. If the interstitial pH is maintained above 7.0 during global ischemia regardless of the method used, recovery of function ranges was 80-95% of preischemic values. The various manuevers, solutions and conditions have shown that ischemic intervals can be increased from 17 to 92 minutes by a single injection of a cardioplegic solution

under very cold conditions. The pH transducer has now been employed in five patients.

Cardiomyopathy

Previous notions of a singular operation for the relief of left ventricular outflow tract obstruction have been discarded. The intraoperative echocardiographic studies of McIntosh and Maron have shown that the classic asymmetric hypertrophy of the cephalad portion of the interventricular septum is present in 70% of patients referred for surgical palliation. A left ventricluar septal myectomy provides good palliation in these patients. However, patients with septal thicknesses less than 18 mm, those with persistent obstruction after a septal myectomy, those with atypical septal morphology and patients with severe mitral regurgitation are best palliated by mitral valve replacement. Consequently 31 patients of a group of 114 have had mitral valve replacement in the past 30 months. Sixteen patients have been restudied by cardiac catheterization, echocardiography, radionuclide angiography, and exercise testing. All but one patient has survived and all but one of the survivors has improved at least one NYHA functional class. The followup will continue to determine the long-term results of this palliative operation in comparison to the group that has septal resection.

Left ventricular septal myectomy (Morrow procedure) continues to be the operation of choice in the majority of patients having surgical palliation in the past 30 months. Sixty-two patients receiving no medications had a resting gradient of greater than 50 mm Hg and the remainder (20) had a lower gradient. Both groups developed similar provakable gradients ranging from 74-115 mm Hg, although patients with high preoperative rest gradients were not provoked. Most had intraoperative echocardiography before and after septal resection. Candidates for the procedure were those that had septal thicknesses greater than 18 mm in the most cephalad 3-4 cm of the interventricular septum. The results show that the operative mortality was 8.5% and the late mortality was 5.3%. Eight patients have had iatrogenic creation of an interventricular septal defect all of whom had coronary artery disease. Resting gradients decreased markedly (93-15 mm Hg) in the high gradient group but most had provokable gradients (31-74 mm Hg). The low gradient group also had provokable gradients (20-37 mm Hg). The level of these gradients did not correlate to symptomatic improvement at six months after the procedure. Ninety percent were a NYHA class of I-II compared to the preoperative status of III-IV. Further studies will be directed toward further refinement of criteria for choice of palliative surgical procedures in an effort to decrease morbidity and mortality especially in those patients with coexisting coronary artery disease.

New Ultrasonic Technologies

The new systems which integrate M-mode, 2-D, pulse and continuous wave Doppler at multiple frequencies have been assessed in a large number of in vivo and in vitro experiments. Additionally, a color encoded ultrasound system has been used for flow field visualization. Twenty-three Newfoundland dogs with genetically occurring subaortic stenosis have been studied. Continuous wave Doppler extrathoracic recording of maximal velocities ranged from 98 to 539 cm/s and correlated with simultaneous hemodynamic gradients of 3 to 123 mm Hg ($r=0.92$). Maximal velocities were used to calculate pressure gradients based on a simplification of the Bernoulli equation and had a correlation coefficient of

0.95 with a SEE of 7.1 mm Hg to the catheterization gradient. The findings are directly applicable to patients with subaortic stenosis.

In other studies, nine ventricular and atrial septal defects 3 to 9 mm in diameter were made. Pulmonary flows increased from 20 to 350% of normal. Color encoded flow mapping accurately detected all defects including multiple ones. This system has proved superior to conventional ultrasound techniques for detection of septal defects. The color flow mapping system has been used to study 21 different types of heart valves placed in sheep. The flow pattern through normal mitral valves has been determined and compared to that through prosthetic valves. Normally, no in-orifice flow disturbances are present and low velocity flow profiles are directed toward the left ventricular apex. All prostheses tested showed moderate to severe flow disturbances. This system permits in vivo evaluation and comparison of normal and malfunctioning native and prosthetic heart valves. Doppler and color flow mapping studies have also been conducted on 23 juvenile baboons in which right ventricular to pulmonary artery conduits had been implanted. These studies were complimented by hemodynamic, angiographic and morphologic data. Doppler measurements accurately predicted peak systolic gradients obtained by cardiac catheterization. It was possible to follow the progression of obstruction within the conduit system over time by use of these ultrasonic technologies. In each case, high correlation to standard invasive methods was achieved. It is now possible to assess children as outpatients instead of using hospitalization and invasive procedures.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 - HL 02714-05 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Evaluation of prosthetic cardiac valve failure in an animal model system

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

Pathology Branch

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD, 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10	5	5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The purpose of this project is to develop an animal model of bioprosthetic cardiac valve failure and to utilize this animal model system to evaluate the pathologic alterations and hemodynamic dysfunction which develops in the valves. We have shown that bioprosthetic valves implanted in juvenile sheep demonstrate the same pathologic alterations of degeneration and calcification as those implanted in humans; however, the development of these alterations is accelerated in sheep as compared to humans. Nearly 500 porcine aortic or bovine pericardial bioprosthetic valves from nine different sources have been implanted in the animal model system to assess the characteristics of the pathologic changes, to compare the alterations in different types of valves, to compare the alterations occurring in valves implanted in the mitral versus the tricuspid positions, and to evaluate valves treated prior to implantation with processes to retard or eliminate the calcification process. Due to these types of observations, clinical trials have been initiated with one new type of bioprosthetic valve. Equally important, if not more so, at least five types of valves have been rejected from consideration for clinical use. The model is additionally being utilized to validate ultrasonic techniques, including color encoded 2-D Doppler, for the characterization of bioprosthetic, mechanical, and synthetic leaflet valve velocity/flow profiles and for the noninvasive detection of valve failure.

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Project Description: Although more than 20 years have passed since the first prosthetic cardiac valves were implanted in humans, the development of an ideal cardiac valve substitute remains a major problem in cardiac surgery. Bioprosthetic cardiac valves have become the valves of choice at many institutions and for certain subgroups of patients, primarily because they do not require chronic anticoagulant therapy. However, it is now apparent that the long-term durability of bioprosthetic cardiac valves is finite; degeneration and calcification of these valves are the major complication of long-term implantation. It is estimated that more than one-half million bioprosthetic valves have been implanted in human patients to date. Of additional concern is the fact that somewhere between 50-100,000 valve replacement operations continue to be performed annually world wide.

An *in vivo*, *in situ* investigational model is essential for the study of the mechanisms of degeneration and calcification in substitute bioprosthetic cardiac valves. For this purpose we have the following criteria in an animal model system developed in domestic sheep (*Ovis aries*): (1) the bioprostheses should be implanted in growing animals to simulate the physiologic conditions in young humans in whom accelerated degenerative alterations of bioprosthetic valves are known to occur; (2) the pathologic alterations should develop within several months after implantation to permit expeditious study; (3) at maturity the animals should not outgrow the bioprosthetic cardiac valves; (4) the animals must be of suitable sizes for standard cardiopulmonary bypass techniques; and (5) the pathologic alterations in the bioprosthetic valves must be similar in the animals and in humans.

During the past four years an animal model system has been developed in juvenile (10-15 week old) sheep which permits early and late studies of host-prosthesis interactions. Over 500 porcine aortic valves (PAV) and bovine pericardial valves (BPV) have been implanted in the tricuspid (TVR) or mitral (MVR) positions. In analyzing the results obtained with valve implantations in this model system, emphasis has been given to: (1) early and late hemodynamic investigations of valve function; (2) pathologic alterations, including localization of calcific deposits; (3) quantitative analyses of valve calcification; (4) evaluations of treatment processes to decrease valve calcification; and (5) use of intracardiac Doppler 2-D echocardiography to study valve function.

Early hemodynamic studies showed anticipated performance for the manufacturer's size 25 mm valves utilized:

	Mean Gradient (mm Hg)	Gorlin Valve Area (cm^2)
TVR - BPV	4.4 ± 0.3 (n = 61)	1.98 ± 0.16 (n = 61)
TVR - PAV	5.3 ± 0.2 (n = 84)	1.64 ± 0.12 (n = 84)
MVR - BPV	7.8 ± 0.3 (n = 153)	1.98 ± 0.07 (n = 153)
MVR - PAV	9.0 ± 0.3 (n = 168)	1.78 ± 0.07 (n = 168)

Cardiac outputs = 2.91 ± 0.07 L/min (n = 446). At the time of explantation (average 5 months) hemodynamic observations reflected pathologic alterations. Pathologic changes resembled those found in humans, including perforations: microemboli; red blood cell, round cell and giant cell infiltrations; fibrous

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sheathing with and without cuspal retraction and commissural fusion; insudation of plasma proteins; cuspal delamination; infection; presence of cotton fragments; strut creep; cuspal abrasion and perforation by sutures; and calcific deposits. Morphologic sites of calcific deposits included cuspal connective tissue and cuspal cells, the muscle shelf and aortic wall of PAV's as well as microthrombi, vegetations and fibrous sheaths. Cartilage and bone formation have also been observed.

Whereas, nonimplanted valves contained 0.87 ± 0.11 mg of calcium per g of tissue dry weight (n = 34), the following results were obtained for standard porcine aortic (PAV) and bovine pericardial (BPV) valves implanted in the mitral (MVR) or tricuspid (TVR) positions:

	N	Calcium content (mg/g)		N	Calcium content (mg/g)
PAV-TVR	40	89.8 ± 11.6	PAV-MVR	90	84.8 ± 0.6
BPV-TVR	21	190.4 ± 49.1	BPV-MVR	77	82.7 ± 0.6

The effects of preimplantation processing by the Carpentier-Edwards PV2 and the Hancock/Extracorporeal T6 treatment to ameliorate calcification were studied. Both of these processes consist of low pressure fixation of the valves and the incorporation of a surfactant. The results were the following:

CALCIUM CONTENT OF CARPENTIER-EDWARDS VALVES IMPLANTED IN THE MITRAL POSITION

Valve Type	n	Calcium Content (mg/g tissue)	
Standard porcine aortic	18	77.9 ± 12.4	
			p < 0.001
PV2 porcine aortic	15	7.6 ± 2.6	
Standard bovine pericardial	18	73.1 ± 10.3	
			p = NS
PV2 bovine pericardial	11	55.2 ± 12.7	

CALCIUM CONTENT OF HANCOCK/EXTRACORPOREAL VALVES IMPLANTED IN THE MITRAL POSITION

Valve Type	n	Calcium Content (mg/g tissue)	
Standard porcine aortic	25	77.1 ± 14.7	
			p < 0.002
T6 porcine aortic	13	10.1 ± 4.9	

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Standard bovine pericardial	12	130.1 ± 5.4	
			p = NS
T6 bovine pericardial	9	109.7 ± 14.0	

Data = mean value ± S.E.M.; p values for standard versus treated valves

In the sheep model system, both processes significantly decreased calcification of porcine aortic valves, but neither process was effective in ameliorating calcification of bovine pericardial valves. Based upon these types of information, one of the treated valves has entered clinical trials; another has been withdrawn from consideration for clinical applicability.

Pilot studies of five types of synthetic leaflet valves have shown superior opening characteristics compared to bioprosthetic and mechanical valves. However, substantial intrinsic regurgitation was demonstrated in two types of copolymer valves; calcification and leaflet tears were shown to develop within five months in two types of polyurethane valves and one type of polytetrafluorethylene valve.

Thus, implanting bioprosthetic valves in young sheep provides an excellent in vivo, in situ means for investigating prosthetic valves' preparation and design.

Proposed Course: On-going studies include comparisons of the various types of valves, comparisons of the alterations occurring in the mitral versus the tricuspid positions, evaluations of methods to ameliorate or eliminate the degenerative processes, and validation of ultrasonic techniques for evaluating pathophysiologic abnormalities produced by the valves.

Presentations:

Jones, M., Eidbo, E.E., Ferrans, V.J.: Bioprosthetic valve calcification: Large animal studies. International Colloquium on Hancock Bioprostheses. Monte Carlo, October 1984.

Jones, M., Eidbo, E.E., Walters, S.M., Ferrans, V.J., Clark, R.E.: Effects of two types of preimplantation processes on calcification of bioprosthetic valves. International Symposium of Cardiac Bioprostheses, London, May 1985.

Publications:

Jones, M., Eidbo, E.E., Ferrans, V.J.: Bioprosthetic valve calcification: Large animal studies. In Andrews, E.J. (Ed.): International Colloquium on Hancock Bioprostheses. King of Prussia, Extracorporeal, 1985, pp. 171-181.

Jones, M., Rodriquez, E.R., Eidbo, E.E., Ferrans, V.J.: Cuspal perforations caused by long suture ends in implanted bioprosthetic valves. J. Thorac Cardiovasc Surg. (in press)

Jones, M., Eidbo, E.E., Walters, S.M., Ferrans, V.J., Clark, R.E.: Effects of two types of preimplantation processes on calcification of bioprosthetic valves.

In Bodnar, E. and Yacoub M. (Eds.): Cardiac Bioprostheses. New York, Yorke Medical Books. (in press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02731-03 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Operative assessment and results of left ventriculomyotomy and myectomy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

Cardiology Branch

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A standard left ventriculomyotomy and myectomy (LVMM) has been performed for relief of left ventricular outflow tract obstruction secondary to idiopathic hypertrophic subaortic stenosis (IHSS) in 412 patients. This report summarizes 82 patients undergoing an LVM&M for resting and provokable gradients since January 1982. An attempt has been made to define criteria for choice of operation, LVM&M vs mitral valve replacement (MVR) based upon septal thickness, distribution of hypertrophy, level of systolic anterior motion (SAM) contact of septum, and concomitant coronary artery disease. Intraoperative 2-D and M-mode echos have been performed on a number of these patients providing precise data utilized intraoperatively. Patients with concomitant CAD have a greater risk for an iatrogenic VSD creation which may be avoided by a modified LVM&M or MVR. Operative mortality is 8.5% and late mortality 5.3%. Hemodynamic data are presented based on preoperative resting gradients $\leq$ 50 mm Hg and $>$ 50 mm Hg and postoperative resting and provokable gradients are compared between these groups. Postoperative hemodynamic studies reveal good relief of resting gradient in most patients but significant provokable gradients remain in some patients. Reoperation has been performed in some patients with persistent symtoms and gradients. Medical therapy is continued in patients with significant gradients regardless of symptomatic status.

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Project Description: Four-hundred twelve patients have undergone operative palliation for resting or provokable left ventricular outflow tract (LVOT) obstruction secondary to idiopathic hypertrophic subaortic stenosis (IHSS). All patients reported had the classic Morrow operation (left ventriculomyotomy and myectomy LVM&M) and the last 82 patients will be summarized. Mitral valve replacement has been performed in an additional 31 patients for treatment of their IHSS. Choice of operative approach is based upon intraoperative echo examination of the septum regarding septal thickness, distribution of septal hypertrophy; i.e., localized or homogeneous and contact point on septum of the anterior leaflet. The preoperative M-mode and 2-dimensional echo may vary depending upon body habitus providing less precise morphology. A post resection echo is also performed which has been helpful in predicting relief of LVOT obstruction. Indications for MVR rather than LVM&M include: (1) septum < 18 mm in thickness; (2) unusual septal morphology, i.e., prominent Seipp's notch or hypertrophy located out of "operative window"; (3) previous LVM&M with persistent symtpoms and gradients; (4) severe mitral regurgitation. The LVM&M is performed as described by Dr. Morrow, but myocardial preservation is now accomplished with cold cardioplegia and iced slush rather than 30°C total body hypothermia and anoxic flaccid arrest.

Patients were placed in two groups based on preoperative hemodynamic data. Group I includes patients with $\geq$ 50 mm Hg resting gradients and Group II includes patients with $\leq$ 50 mm Hg resting gradients, who are generally recommended for operation because of significant provokable gradients.

Group I - Patients with > 50 mm Hg Resting Gradients ± CAD

Sixty-two patients, 31 males (mean age 48.1 yrs ± 15.9 SD) and 31 females (mean age 50.7 yrs ± 17.6) having resting gradients $\geq$ 50 mm Hg have undergone an LVM&M for relief of LVOT obstruction secondary to IHSS. All were Functional Class III-IV prior to operation. There were 6 early deaths (<30 days: 9.7%) and 3 late deaths (>30 days: 5.4%). Early deaths were attributed to low cardiac output (n=3); iatrogenic VSD and low cardiac output (n=1); iatrogenic VSD requiring reoperation leading to death (n=1); and mediastinitis (n=1); late deaths to arrhythmia (n=2) and sepsis (n=1). There were a total of 3 VSD's in this group; all had concomitant CAD. Eleven of 62 patients (17.7%) had concomitant CAD. Other operations performed included aortic valve replacement (n=3); AVR + CABG (n=1); tricuspid valve replacement (n=1) and closure of iatrogenic VSD early (n=2); late (n=1).

PRE AND POSTOPERATIVE HEMODYNAMIC DATA

	Pre (n)	Post (n)
PAW	16.2 ± SD 6.4 (n = 28)	13.9 ± 5.8 (n = 28)
LVEDP	17.5 ± 7.7 (n = 35)	15.5 ± 5.8 (n = 35)
GRADIENTS		
Rest	93.1 ± 30.8 (n = 42)	14.9 ± 22.8 (n = 42)
		0 gradient n = 24

*Valsalva		
Complete data	109.3 ± 25.8 (n = 19)	30.8 ± 24.1 (n = 19)
Incomplete data (postop only)		58.9 ± 35.6 (n = 20)
AmylNitrite		
Complete data	100.6 ± 19.8 (n = 9)	39.3 ± 30.5 (n = 9)
Incomplete data (postop only)		62.2 ± 33.8 (n = 29)
Isoproterenol		
Complete data	115.0 ± 27.7 (n = 5)	62.0 ± 41.3 (n = 5)
Incomplete data (postop only)		73.5 ± 46.0 (n = 30)
Cardiac Output	4.6 ± 1.4 (n = 26)	4.6 ± 1.6 (n = 26)
Cardiac Index	2.5 ± 0.8 (n = 26)	2.5 ± 0.8 (n = 26)

* Patients with resting gradients ≥ 100 mm Hg not provoked

Group II - Patients with < 50 mm Hg Resting Gradients ± CAD

Twenty patients, 14 males (mean age 47.9 ± 18.5) and 6 females (mean age 52.7 ± 7.9) were found to have resting gradients ≤ 50 mm Hg and underwent LVM&M. Six patients (30%) had concomitant CAD. There was one early death (5.0%) secondary to persistent bleeding and hypotension in a patient who had undergone a previous CABG and one late death (5.3%) attributed to congestive heart failure in a patient with a dilated cardiomyopathy with only a provokable gradient. Morbidity included 4 iatrogenic VSD's (2 patients had CAD), 3 closed at initial operation (1 pt died) and one occurring late closed at 7 days (pt alive); 1 patient required a pacemaker for complete heart block; 1 patient developed mediastinitis; 1 patient developed a compartment syndrome of leg with no apparent etiology.

PRE AND POSTOPERATIVE HEMODYNAMIC DATA

	Pre (n)	Post (n)
PAW	13.0 ± 8.6 SD (n = 11)	10.0 ± 4.1 (n = 11)
LVEDP	15.3 ± 7.7 (n = 14)	14.1 ± 6.7 (n = 14)
GRADIENTS		
Rest	17.6 ± 18.5 (n = 14)	3.2 ± 6.2 (n = 14)
0 gradient	n = 6	n = 10

*Valsalva	73.6 ± 35 (n = 11)	19.5 ± 19.6 (n = 11)
Amyl Nitrite	86.9 ± 23.9 (n = 12)	28.9 ± 19.5 (n = 12)
Isoproterenol	110.6 ± 24.8 (n = 9)	36.9 ± 34.0 (n = 9)
Cardiac Output	4.9 ± 1.1 (n = 13)	4.5 ± 1.2 (n = 13)
Cardiac Index	2.6 ± 0.6 (n = 13)	2.3 ± 0.6 (n = 13)

* Patients with resting gradients $\geq$ 100 mm Hg not provoked

Patients with resting gradients > 50 mm Hg may expect excellent relief of LVOT obstruction following the Morrow procedure but may have significant residual provokable gradients. Medical therapy is recommended in patients having $\geq$ 50 mm Hg provokable gradients. Symptomatic improvement may not reflect hemodynamic data in that a majority of patients are Functional Class I-II at 6 months. Patients undergoing operation for provokable gradients will have smaller postoperative gradients but a small percentage will provoke to 50 mm Hg. Ten patients with persistent symptoms and demonstrated hemodynamic obstruction who cannot be managed with beta or calcium channel blockers have been reoperated upon having mitral valve replacement. All patients survived MVR and improved symptomatically. Two had persistent chest pain and were found to have limited coronary flow demonstrated via a coronary sinus flow study and are being treated with calcium channel blockers.

Proposed Course:

Iatrogenic echos will be continued to further define the spectrum of septal morphology to allow tailoring of the operative approach for maximal relief of LVOT obstruction at the lowest operative risk. Patients who continue to be symptomatic following an LVM&M will be treated medically and if not improved MVR will be recommended.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01HL 02733-02 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mitral valve replacement in selected patients having IHSS

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

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LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD, 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Mitral valve replacement (MVR) has been performed on 31 patients as primary or secondary treatment of resting and/or provokable left ventricular outflow tract obstruction (LVOT) secondary to idiopathic hypertrophic subaortic stenosis (IHSS). Indications for MVR include: (1) septal thickness < 18 mm; (2) persistent LVOT obstruction after an adequate left ventriculomyotomy and myectomy; (3) atypical septal morphology; and (4) severe mitral regurgitation secondary to ruptured chordae tendineae or papillary muscle. Intraoperative echo has provided very useful images of the septal morphology which allows selection of patients for MVR. There has been 1 (3.2%) operative death secondary to hepatic and renal failure and no late deaths. Symptomatic improvement to functional class I or II has occurred in 92.8% of 16 patients returning for postoperative evaluation. Excellent hemodynamic relief of resting and provokable gradients have been demonstrated. Long-term follow-up of these patients will be necessary to assess mortality and morbidity compared to the well known results of left ventriculomyotomy and myectomy used to palliate patients in this institution for 25 years

f 25

Project Description: Left ventriculomyotomy and myectomy (LVMM) has become the procedure of choice in patients having LVOT obstruction secondary to IHSS in this clinic. This procedure has been utilized as primary treatment in 430 patients and long-term functional and hemodynamic results have been well established over 25 years. The age of our adult population recommended for operation is increasing secondary to the variety of beta blockers and calcium channel blockers currently available for treatment of IHSS. Intraoperative ECHO's are providing an increasing appreciation of the spectrum of septal morphology that occurs in this disease and have provided the rationale and selection of patients to be palliated with an MVR rather than the standard LVM&M. Current indications for MVR as primary operative treatment are: (1) septum 18 mm or $\leq$ in area of resection (n=11 patients); (2) atypical septal morphology (n=7); (3) mitral regurgitation related to endocarditis or ruptured chordae tendineae or papillary muscle secondary to ischemic disease (n=3); and secondary treatment for (4) persistent obstruction following an adequate LVM&M (n=10).

Sex and age distribution of these 31 patients is 16 males, mean age 46.9 years (range 29-71) and 15 females, mean age 44.5 years (range 16-68). Sixteen patients have returned for postoperative clinical and hemodynamic study approximately 6 months after operation. Preoperative functional class (New York Heart Association) was III for 11 patients and IV for 5 patients. Mitral valve replacement was performed using standard extracorporeal techniques and cold cardioplegia for myocardial protection. The Bjork-Shiley convexo-concave prosthesis was used in most patients unless there was a contraindication to anticoagulants; then a Hancock bioprosthesis was selected. Because of the prominent left ventricular hypertrophy (LVH) and normal mitral valve annulus size, smaller prostheses (23-27 mm) were implanted. Two of 31 patients required concimitant CABG in addition to MVR. There was 1 (3.2%) early death ($\leq$ 30 days) secondary to hepatic and renal failure of unexplained etiology and no late deaths.

Postoperative functional class in the 16 patients evaluated was I in 7 patients; II in 7 patients, and 2 patients in class III. Therefore 92.8% of patients had improved to functional class I or II status following MVR. There have been no emboli, anticoagulation complications or prosthetic dysfunction in returning patients. A perivalvular leak occurred in 1 patient and required repeat operation for closure.

Hemodynamic results have been excellent and consistent and are summarized below by indications for MVR.

Hemodynamic Summary of Patients Having MVR for Treatment of IHSS (Pre/Postoperative)

	PAW	LVEDP	Rest	Valsalva	Gradients Amyl Nitrate	Isuprel	CO//CI
Group I (n=5) (thin septum)	15/14	16/11	57/11	105/4	110/21	130/28	4.7/4.7//2.7/2.5
Group II (n=6) (prev. LVM&M)	11/13	15/13	40/5	62/9	73/14	114/15	5.3/5.4//2.9/2.9
Group III (n=3) (atypical septal morphology)	14/14	15/13	97/5	110/12	-/15	-/23	4.3/5.4//2.4/2.

F26

Group IV (n=2) Data incomplete
(severe MR - etiology other than IHSS)

Thus, MVR has provided excellent relief of resting and provokable gradients. In spite of the smaller sizes of prostheses implanted, significant prosthetic stenosis has not been demonstrated and PAW's remain in high normal range. MVR is indicated in patients having septums 18 mm or $\leq$ to avoid an iatrogenic VSD and in previous patients having had an LVM&M for maximal relief of gradients.

Proposed course: Long-term follow-up will be necessary to establish survival, particularly deaths which may be prosthesis related and long-term symptomatic relief. Survival curves will also be derived concerning complications such as emboli, anticoagulation complications and prosthetic malfunction requiring reoperation for comparison to similar curves for patients undergoing LVM&M.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02735-02 SU

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mitral valve replacement with and without chordal excision

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

Charles L. McIntosh, M.D., Senior Surgeon, Surgery Branch, NHLBI
Michael Jones, Senior Surgeon, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD, 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The purpose of this clinical trial is to test the hypothesis that the mitral valve apparatus (leaflet, chordae tendineae and papillary muscles), if left intact at the time of valve replacement, will prevent the nearly uniform postreperfusion left ventricular dilation and low cardiac output associated with surgical treatment of mitral insufficiency of long duration.

The specific aims are: (1) measure LV hemodynamics and dimensions with ultrasound prior to and after cardiopulmonary bypass in the OR; (2) replace the mitral valve in patients with pure mitral regurgitation with and without resection of the entire mitral valve apparatus; (3) determine immediate postoperative hemodynamic characteristics of each group; (4) analyze 6 month and 3 year results in terms of exercise capacity, LV dimensions, and hemodynamic criteria.

The rationale of this investigation is the hope that if the immediate postbypass course of patients receiving mitral valve replacement for long standing mitral insufficiency can be altered by the mechanism of preventing left ventricular dilation by maintaining the innate physical structures of the left heart, operative mortality and support measures may be reduced and long-term benefits may accrue.

The results in five patients have been excellent. Two patients have had bioprostheses and 3 patients have had St. Jude prostheses. Matched paired patients having mitral valve replacement during the same interval have had subtle changes of left ventricular dysfunction in the postoperative interval which appear typical and different than the experimental group. Six month data will be obtained soon.

F28

Project Description: This clinical trial tests the hypothesis that the chordal-papillary muscle annular ring and leaflet apparatus is important in left ventricular mechanics associated with mitral insufficiency. The evidence for this is divergent and subtle dating back to studies by Lillehei in 1960. Low cardiac output was associated more often when the papillary muscles were excised than when these structures were left intact. Animal data suggests that in normal ventricles, excision of the entire valvular apparatus and mitral valve replacement results in an increase of 20-40% systolic and diastolic dimensions at normal to low preload. A clinical trial in Hannover, West Germany and Toronto, Canada showed similar good results by maintaining continuity of these structures.

Mitral valve replacement in patients is performed in standard manner. The only exception is that little if any of the mitral leaflets tissue and/or chordae tendineae are resected. The suture techniques are identical whether or not the valve is removed. Pressure, flow and ultrasonic studies are used before, and after valve replacement with the chest open. Hemodynamic studies are performed in the intensive care unit for 24-72 hours. Six months after surgery, the patients are to be restudied by cardiac catheterization, nuclide angiography, and ultrasonic studies. Five patients have had this procedure; three having a St. Jude bileaflet mechanical prosthesis and two a Hancock bioprosthesis. The results in four patients have been excellent with little to no ventricular dilation, increase in cardiac output, and no requirement for any type of catecholamine therapy. Five patients, matched for age, sex, and extent of disease have all had subtle difficulties after mitral valve replacement for mitral insufficiency with complete excision of the valve.

Proposed Course: The study is to continue.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02740-02 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*

Coronary vascular tone after coronary bypass operations

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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COOPERATING UNITS *(if any)*

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LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This clinical study tests the hypothesis that coronary blood flow dynamics of patients may be altered in the immediate interval after a coronary artery bypass procedure which may be deleterious to the patient's future course if unrecognized. Six patients have had complete studies. A thermal dilution catheter is placed into the coronary sinus and threaded to the great anterior vein. Ports are at the tip and more proximal for mixed effluent sampling. Blood samples are used to determine regional oxygen consumption, acid base balance, lactic acid, pyruvate, creatine kinase and lactic dehydrogenase iso-enzyme concentrations. Complete hemodynamic evaluations are performed in serial fashion for 6-8 hours after operation. The results to date show wide variation of response to blood flow augmentation. In some, no significant changes in coronary vascular resistance, cardiac output, or any of the biochemical variables occur. Other patients show changes which can be related to less than optimal contractility and systemic flow.

f 3 0

Project Description: Adult males greater than 40 and less than 70 years of age who require more than one coronary artery bypass graft were selected. Informed consent is obtained. Prior to the induction of anesthesia, appropriate catheters are inserted into the left radial artery, superior vena cava, pulmonary artery, and coronary sinus. The last is advanced to the level of the great anterior cardiac vein which parallels the left anterior descending coronary artery. Complete studies are obtained in the catheterization laboratory, after induction of anesthesia, prior to and after cardiopulmonary bypass, and every hour thereafter for six to eight hours. Regional oxygen consumption, pH, pCO_2, pO_2, arterial and venous saturations, coronary and systemic blood flow, lactate, pyruvate, creatine kinase and lactic dehydrogenase isoenzyme concentrations are determined by serial sampling of various blood pools. Systemic, pulmonary, and coronary artery resistances are calculated as are left and right ventricular stroke work. Data analysis consists of time-concentration plots of the major variables.

Proposed Course: This study is to continue until 15-25 patients have had complete data.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02742-02 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Assessment and use of new ultrasonic technologies

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Lilliam M. Valdes-Cruz, M.D., Guest Worker, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institution, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues XXX (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

New modes of Doppler ultrasound and new signal conditioning of the received sonic specta permit accurate assessment of mean and peak velocities of intracardiac blood flow. Color encoded, two dimensional Doppler permits qualitative and quantitative evaluations of entire flow field velocity patterns. Studies utilizing these technologies include the following: (1) Quantitation of gradients in Newfoundland dogs with subaortic stenosis; (2) Comparison of ultrasound Doppler with laser Doppler anemometry; (3) Studies of intracardiac shunt flow; (4) Doppler color flow mapping of prosthetic mitral valves; and (5) Quantitation of obstruction and regurgitation in right ventricular to pulmonary artery conduits with and without prosthetic valves. Studies in patients, both intraoperatively and postoperatively, have been initiated for the assessment of operations for valvular lesions and congenital cardiac defects.

f32

Project Description: The development of continuous wave Doppler systems with multiple frequencies integrated with pulsed, 2-D and M-mode in one device has opened a new field of noninvasive accurate quantitation of a variety of pathophysiologic hemodynamic conditions. This technology may permit serial assessments of the palliative benefits, or lack thereof, in a host of operations for congenital and acquired heart disease. The benefits of continued exploration of these technologies are (1) accurate noninvasive determination of improvement or deterioration of diseased native or prosthetic heart valves and conduits; (2) avoidance of repeated cardiac catheterizations; and (3) more frequent observation in the outpatient setting as opposed to costly hospitalization. In addition color encoded, two dimensional Doppler permits in vivo velocity/flow field visualization which should add substantial understanding of the hemodynamic pathophysiology of prosthetic heart valves.

Twenty-three Newfoundland dogs with subaortic stenosis were studied by closed chest Doppler interrogation of aortic velocity from an apical view of the left ventricular outflow tract simultaneously with measurements of pressure gradients during cardiac catheterization. Continuous mode Doppler interrogations were used with two-dimensional echocardiographic guidance to compare the Doppler derived maximal velocities with pressure gradients across the obstruction at rest and after provocation with amyl nitrite inhalation and isoproterenol infusion. The maximal velocities recorded by Doppler ranged from 98 to 539 cm/s and correlated with hemodynamic gradients ranging from 3 to 123 mm Hg (r=0.92, SEE=37 cm/s). Doppler velocities were converted to gradients using a simplification of the Bernoulli relation (gradient = 4 x maximal velocity^2); the resulting Doppler derived gradients also correlated closely with the catheterization measured pressure gradients (r = 0.95, SEE = 7.1 mm Hg). The predictive capability of Doppler echocardiography for estimating the pressure gradients across subaortic obstruction in this group of dogs with a spectrum of congenital heart disease similar to that found in human beings was thus validated. These observations indicate that Doppler methods have clinical applications in human patients with subaortic stenosis.

Peak flow velocities recorded by continuous wave and high pulsed repetition frequency Doppler were compared to those measured with a triple beam helium-neon laser Doppler anemometry system in an in vitro pulsatile flow model of valvular pulmonic stenosis. Bovine pericardial valves were mounted in a glass simulation of a pulmonary artery with a bifurcation attached to a pulse duplicator. Velocities measured from 2% cornstarch particle solution (viscosity = 3.5 cP at 32°C) were compared to instantaneous gradients. For 37 hemodynamic gradients ranging from 2 to 123 mm Hg, continuous wave Doppler (r = 0.99, SEE = 17.6 cm/s) and high pulsed repetition Doppler (r = 0.99, SEE = 15.5 cm/s) correlated highly with laser Doppler anemometry derived velocities. Using the modified Bernoulli equation: gradient = 4x maximal velocity^2, continuous wave and high pulsed repetition Doppler predicted gradients well - for continuous wave, r = 0.98, SEE = 7.8 mm Hg; for high pulsed repetition, r = 0.98, SEE = 6.3 mm Hg; for laser Doppler anemometry, r = 0.97, SEE = 8.2 mm Hg. Compared to laser Doppler anemometry, both continuous wave Doppler and high pulsed repetition frequency Doppler accurately measured actual velocities, in this idealized in vitro model. These results are a "best case" accuracy estimate for gradient using the Bernoulli relationship for application in clinical studies.

Nine ventricular and nine atrial septal defects varying between 3 and 9 mm in diameter were made in dogs to study flow imaging across the defects using color encoded Doppler flow mapping techniques. Postmortem examinations confirmed that the smallest atrial septal defect was 3 mm in diameter and the smallest ventricular septal defect was 2.6 mm in diameter. Pulmonary to systemic blood flow shunt ratios calculated from aortic and pulmonary artery flows determined electromagnetically ranged from 1.2/1.0 to 3.0/1.0 for atrial septal defects and from 1.1/1.0 to 4.5/1.0 for ventricular septal defects. All atrial septal defects were imaged and flow through them recorded as jets crossing the atrial septum from left to right. Flows through the smaller defects associated with higher interatrial gradients were easily detected. No flow was seen through the 2.6 mm ventricular septal defect; flow was imaged across 3 mm ventricular septal defects, although the defects were too small to be imaged clearly by two-dimensional echocardiography alone. Five ventricular septal defects larger than 3 mm were clearly imaged anatomically and Doppler determined flow through them recorded. The two-dimensional Doppler flow mapping system also was capable of resolving the presence of multiple small atrial and ventricular septal defects, which should be of substantial clinical and surgical importance. Therefore, this new technology appears to have high resolution capabilities in detecting flow across single or multiple septal defects even when they are too small to detect by conventional ultrasound imaging techniques.

Thirty-five Doppler color flow mapping studies of 21 different types of clinical and preclinical valves were performed after implantation in the mitral position in sheep. Twenty-nine studies were performed immediately after implantation and six were performed 20 weeks after implantation. The valves, obtained from 10 manufacturers, included 10 bovine pericardial valves, 8 porcine aortic valves, 9 mechanical valves and 8 synthetic leaflet valves. Color Doppler velocity/flow profiles were imaged in real-time and with electrocardiographic gating. Native mitral valves had no in-orifice flow disturbances and laminar, low velocity/flow profiles directed toward the left ventricular apex. Bovine pericardial and porcine aortic bioprosthetic valves had high velocity, turbulent, eccentric jets in diastole and stagnation associated with flow separation beneath the leaflets in systole. Major and minor orifice velocity/flow disturbances were imaged for all mechanical valves. Synthetic leaflet valves had velocity/flow profiles resembling native mitral valves. Other abnormalities observed included "lazy leaflets", perforations, perivalvular and intrinsic regurgitation, and stenoses due to calcification. These types of velocity/flow patterns were similar to those obtained with in vitro color Doppler and laser Doppler anemometry. Doppler color flow mapping permits in vivo evaluation and comparison of normally functioning and malfunctioning prosthetic mitral valves.

Hemodynamic, angiographic, and morphologic studies of 23 juvenile baboons in which right ventricular to pulmonary artery conduits had been implanted for 12 months documented that fibrous peels produce obstruction, and fusion of the valve cusps to the conduit walls produce regurgitation. Thirty-two simultaneous hemodynamic and Doppler studies were performed at different time intervals in 12 baboons. Catheterization peak systolic gradients ranged from 13 to 205 (mean 66) mm Hg. Doppler maximal velocities ranged from 1.2 to 6.4 (mean 3.9) m/sec. Maximal velocities correlated with catheterization peak systolic gradients: catheterization gradient = 1.16 (4 x maximal velocity^2) + 1.3; r = 0.96; SEE = 3.0. Longitudinal Doppler studies showed progression of obstruction from 10 to

to 44 mm Hg at 12 and 18 months in one animal, and from 60 to 90 to 160 mm Hg at 12, 18, and 24 months in another animal. Serial studies performed from the time of implantation upon 6 baboons showed an average gradient of 16 (range 9 to 25) mm Hg immediately after implantation, of 33 (range 26 to 39) mm Hg after 3 months, and of 54 (range 38 to 69) mm Hg after six months. Color Doppler velocity/flow studies were performed on 3 conduits, one each at 3, 6 and 24 months. At 3 months no important abnormalities were apparent; at 12 months conduit obstruction and regurgitation were visualized; and at 24 months conduit obstruction and regurgitation associated with tricuspid regurgitation and an atrial right-to-left shunt were imaged. Thus, noninvasive, ultrasonic techniques should have clinical applications in following patients after Rastelli types of operation.

Proposed Course: These projects will continue and new uses explored to make possible clinical assessments of cardiac abnormalities. A collaborative study has been initiated with Dr. Ajit P. Yoganathan, Biofluid Dynamics Laboratory, Georgia Institute of Technology, to compare in vivo and in vitro ultrasound Doppler techniques and laser Doppler anenometry studies of prosthetic cardiac valves.

Publications:

Valdes-Cruz, L.M., Jones, M., Scagnelli,S., Sahn, D.J., Tomizuka, F.M., Pierce, J.E.: Prediction of gradients in fibrous subaortic stenosis by continuous wave two-dimensional Doppler echocardiography: Animal studies. J Am Coll Cardiol 5: 1363-1367, 1985.

Tomizuka, F., Valdes-Cruz, L.M. Yoganathan, A., Sahn, D.J., Main, J.C., Swensson, R.E., Jones, M., Eidbo, E.E.: Comparison of continuous wave and high PRF ultra sound Doppler velocities to laser Doppler anemometry in an in vitro pulsatile flow model of valve pulmonic stenosis: Accuracy for gradient prediction. Circulation 70: 11-282, 1984.(abstr)

Sahn, D.J., Valdes-Cruz, L.M., Jones, M., Eidbo, E.E., Tomizuka, F., Elias, W., Dalton, N., Hagen-Ansert, S., Sherman, F., Swensson R: Detection of flow through experimentally created atrial and ventricular septal defects in animal models by color coded Doppler flow mapping techniques: Spatial accuracy and sensitivity of the technique. J Am Coll Cardiol 5: 453, 1985.(abstr)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02743-02 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

The development of a specific immune tolerance model in rhesus monkeys

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, end institute affiliation)*

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COOPERATING UNITS *(if any)*

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LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

It has been shown both experimentally by Medawar and in nature by Owen that exposure of various mammalian species to foreign antigens during fetal or neonatal life can result in permanent specific immunological tolerance to those antigens. Burnet proposed the clonal selection theory of acquired immunity, which explains the tolerance phenomena by the deletion of foreign antigen-specific lymphocytes when they are exposed to their respective antigens while the lymphocytes are in an immature state. These observations led to the hypothesis that an adult animal, modified so as to have a fetal-like immune system and subjected to a foreign graft transplant, would develop permanent specific immunological tolerance to the graft. A juvenile rhesus monkey model has been developed over the past three years to test the hypothesis. Briefly, the interventions are: 1) recipient bone marrow harvest, 2) T lymphocyte removal from the marrow by physical (E-rosette) and immunological (antibody plus complement) methods, 3) total body irradiation of the recipient with a myeloablative dose to eliminate all immunologically competent cells, 4) reinfusion into the recipient of the T lymphocyte-depleted marrow to salvage the recipient from the radiation, and 5) transplantation of an antigenically-mismatched heterotopic heart allograft. Each experiment includes a treated recipient paired with an appropriate control animal. The major endpoint is time to graft rejection, determined by loss of electrocardiographic activity and confirmed by histopathological examination. Other responses being followed are the time course of return of immunological function, and tests of specific immunological tolerance. Only recently have preliminary results become available.

f36

Project Description: The work of the past twelve months falls into the following categories: 1) tolerance induction-related methodology, 2) in vivo immune testing, and 3) in vitro immune testing.

Concerning tolerance induction: 1) a sheep erythrocyte-rosette depletion step has been added to the bone barrow T lymphocyte depletion protocol; 2) the final monoclonal antibody combination consisting of antibodies 9.3, 51.1, 66.1, and 22 (the latter a pan-T cell reagent replacing 9.6) has been selected for use in the bone marrow T lymphocyte depletion protocol; 3) two T cell quantitative assays have been worked out for assessment of residual T cell content of depleted bone marrow, namely fluorescence-activated cell sorting (FACS, capable of detecting 1% T cell contamination) and clonogenic growth assay (cumbersome, but capable of detecting 0.001% T cell contamination); 4) RhLA Class I (serologically-defined) antigen typing of the rhesus monkey colony has been performed with rhesus typing sera and complement in a standard micro-lymphocytotoxicity assay; 5) RhLA Class II (lymphocyte-defined) antigen typing of the colony has been performed in mixed lymphocyte reaction (MLR) assays; 6) the monkey colony has been arranged in maximally mismatched donor-recipient pairs based on differences in sex, kinship, Class I and Class II differences, and geographical origin of the parents; and 7) further heterotopic heart transplant procedures have been performed in cats for finalization of the operative protocol.

Concerning immune testing, it was felt desirable to assess the time course of return of immune function post-radiation/transplantation with regard to a correlation with graft rejection and a comparison between recipients of T lymphocyte-depleted and undepleted bone marrow. Because there is an inconsistent relationship between the results of in vivo and in vitro immune functional assays, the use of both types would be preferable. A series of experiments in a separate group of rhesus monkeys has resulted in an in vivo immune assay consisting of immunization against Candida, Trichophyton, and Tetanus antigens (emulsified with incomplete Freund's adjuvant), and testing for responsiveness with standard delayed-type hypersensitivity skin testing. Although consistent brisk responsiveness has been achieved in animals immunized against all three antigens simultaneously, much less consistent results have been obtained when only one immunizing antigen has been used. The following in vitro immune assays have been worked out for the rhesus monkey: 1) FACS to quantitate the distribution of T pan, Thelper, Tcytotoxic/suppressor, and B lymphocytes in peripheral blood (PB); 2) concanavalin-A stimulation of PB lymphocytes to assess global T cell function; 3) xenogeneic-stimulator (human lymphoblastoid cells) MLR assay to assess Thelper function; and 4) xenogeneic-target (as above) cell-mediated lympholysis assay to determine PB Tcytotoxic function.

The tolerance induction protocol incorporates the described methodology, as well as that which was developed earlier, including marrow harvesting, total body irradiation, and peri-radiation care. Briefly, the steps are: 1) marrow harvest on day-2 with T cell depletion (treated group) or no modifications (control group); 2) TBI 600 rads on days-1 and 0; 3) marrow transplant on day 0; and 4) heterotopic heart transplant on day 1. Three experiments, each consisting of a treated animal paired with a control animal, have been performed

since March 1985. The results are too preliminary to be reported, and some problems have been encountered; nonetheless, progress is being made.

Proposed Course: The project will be continued over the next 12 months.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02746- 02 SU

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The effect of hypothermia and cardioplegic solutions on intramyocardial pH

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
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Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1.0	1.0

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Unknown is the effect of hypothermia or various cardioplegic solution compositions at various temperatures on interstitial myocardial pH during global ischemia and reperfusion. Hydrogen ion flux is very rapid in ischemic tissue and especially so during the initial 30-90 seconds of reperfusion. An NIH developed fiberoptic system using membrane captured phenol red can measure pH in tissues continuously. These studies were designed to test the hypothesis that the exponential rate of rise of hydrogen ion concentration in ischemic myocardium is altered substantially by hypothermia because of known pK_a changes in water as a function of temperature. Further, the imidazole component of red blood cell buffer is known to interact but is eliminated in these studies by frequent washout of the coronary system. The data show a marked blunting of the time - pH decay with hypothermic and cardioplegic solutions. No significant difference was demonstrated in recovery of function between control (pH 7.4) and pH 7.0 groups. Recovery was significantly depressed at terminal ischemia pH values of 6.8 and 6.6. Recovery values were equal at all pH values, although hypothermia at 25°C provides a 2-3 fold increase in time to reach a specific pH value. Cardioplegic solution infusion resulted in little time improvement unless under high pressure and very cold. Prolongation to 92 min was achieved with a single infusion of 1°C solution to reach a pH value of 6.8. The system has been used on five patients.

PHS 6040 (Rev. 1/84) GPO 904-917

Project Description: Ninety-two sheep were used in these studies. After the induction of anesthesia, intubation, volume ventilation, and left thoracotomy arterial, left atrial, left ventricular and pulmonary artery catheters are placed. pH and temperature sensors are placed in the septum and left ventricular free wall. Cardiac output is measured by thermal dilution. Left ventricular stroke work (LVSW) versus mean left atrial pressure (LAP) curves are generated by volume loading with fresh filtered whole blood to LAP of 5-15 mmHg. The left femoral artery is cannulated with a 14 Fr. cannula and the pulmonary artery with a 28 Fr. cannula. Hypothermic cardiopulmonary bypass (CPB) is instituted. A LV vent is placed through the left atrial appendage. Control animals (n = 5) undergo one hour of CPB at 24°C without ischemia. Three additional groups of animals had the aorta crossclamped and the pH allowed to fall to 7.0, 6.8, or 6.6. Myocardial temperature during global ischemia is similar in all groups. Animals are weaned from CPB without inotropic agents. One hour after termination of CPB, left ventricular function curves are again determined. The percent recovery of LVSW is derived from the ratio of the integrals of the pre and postbypass stroke work curves. The preliminary data are shown below.

NORMOTHERMIC (38°C) GLOBAL ISCHEMIA

pH Group	n	Ischemic Time (min)	dpH/dt	%Recovery LVSW
7.4	6	0	0	95 ± 3
7.0	6	10.8 ± 0.7	.039 ± .003	84 ± 4
6.8	11	15.6 ± 0.8	.039 ± .002	47 ± 5
6.6	6	23.8 ± 3.2	.040 ± .005	32 ± 8

HYPOTHERMIC (38°C) GLOBAL ISCHEMIA

7.4	5	0	0	96 ± 3
7.0	6	20.3 ± 1.8	.022 ± .002	88 ± 6
6.8	7	46.1 ± 6.3	.014 ± .002	58 ± 7
6.6	5	65.1 ± 7.6	.014 ± .002	28 ± 6

---

pH Group	n	CP Dose	Port	Flow (ml/min)	Aortic Root Pressure
6.8	6	0	--	--	--
6.8	5	1	19g	180	90 mm Hg
6.8	6	2	19g	180	90 mm Hg
6.8	5	1	16g	450	130 mm Hg
6.8	5	2	14g	450	130 mm Hg
6.8	7	0			
6.8	5	1	14g	450	130 mm Hg

pH Group	n	Temp	Defib	Ischemic Time (min)	dpH/dt	%Recovery LVSW
6.8	6	38	6/6	17.1±1.2	.036±.003	49±9.5
6.8	5	38	5/5	17.3±1.0	.034±.002	48±7.3
6.8	6	38	2/6	19.4±1.4	.031±.003	47±6.9
6.8	5	38	3/5	23.7±0.9	.024±.001	46±6.8
6.8	5	38	1/5	27.9±0.8	.025±.001	45±9.7
6.8	7	25	4/7	46.1±6.3	.014±.002	59±7
6.8	5	25	0/5	52.2±4.2	.011±.002	49±6.3

The data show that 34% reduction in temperature significantly slows the rate of decline of pH and extends the ischemic interval to a specific pH value by a factor of 2-3. Aortic root pressures of 130 mm Hg during cardioplegic solution infusion provided added protection. Preliminary data with solution temperatures of 1°C show the ischemic interval to pH 6.8 can be prolonged to 92 min.

Proposed Course: The pH device will be used in the laboratory to test the effects of various additives to cardioplegic solutions.

Publications:

Ribakove, G.H., Glassman, L.R., Voigtlander, J.P., Takach, T.J., Jones, M., and Clark, R.E.: On-line fiberoptic pH microprocessor system predicts recovery of function following global myocardial ischemia. Surgical Forum. XXXV:275-277, 1984.

Takach, T.J., Glassman, L.R., Voigtlander, J.P., and Clark, R.E.: Continuous measurement of intramyocardial pH: Correlation to functional recovery. Ann Thorac Surg. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02747-02 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Amiodarone and myocardial preservation in hypertrophied myocardium

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Thomas J. Takach, M.D., Clinical Associate, Surgery Branch, NHLBI

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD, 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The hypothesis tested was that amiodarone, a potent cardiotonic agent with unknown mechanisms of action, has a dose related effect on protection of hypertrophied myocardium. The working isolated rat heart preparation was used at 37°C. Filling pressures and afterload were maintained constant before and after 30 minutes of global ischemia. One injection of 12 ml of normal saline only or containing 0.125-12 mg of amiodarone was given at the onset of ischemia. The determinants of recovery were heart rate, aortic flow, and coronary blood flows. The results show a narrow band width dose response with approximately 40% recovery at 0.125 and 0.5 mg dosages. Maximal recovery of 70% of aortic flow was found at 0.25 mg dose. Higher doses produced decremental values of recovery. This study demonstrates that with single dose use at normothermia, amiodarone may be a useful adjunct for cardioplegic solutions but has a narrow dose-response range. Hypothermic drug treated hearts had significantly ($p < .05$) enhanced recovery of function vs. normothermic drug treated hearts but differences between hypothermic control and normothermic controls were not significant ($p < .10$) indicating possible synergy or addition between hypothermic and drug effects. It is concluded that amiodarone ameliorated the deleterious effects of global ischemia in hypertrophied rat hearts at normothermic and moderately hypothermic temperatures.

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Project Description: The purpose of these experiments was to test the efficacy and toxicity of a new investigational cardiotonic agent, amiodarone, for its myocardial protective effects during global ischemia at 37°C. The working isolated rat heart was used to assess dose-response characteristics. Seventy-eight rat hearts from a strain of spontaneously hypertensive rats were used. The hearts were rapidly excised and perfused in Langendorf fashion with oxygenated Krebs-Henseleit solution. After various catheters and transducers were applied, the preparation was converted to the working mode by providing inflow through the left atrium. Aortic flow was measured electromagnetically and the coronary sinus flow by timed volume collections. Temperature, left atrial filling pressure and aortic resistance were maintained constant. After a 15-30 min interval of steady state conditions, all flow was stopped. The aortic outflow cannula clamped, and 12 ml of 37°C normal saline with or without amiodarone was injected into the aortic root in 90 sec. Dosage range was 0.125 to 12 mg. After 30 min the heart was reperfused in retrograde manner and then converted to the working mode. Aortic flow was the primary indicator of recovery.

In other experiments at initiation of ischemia, each heart received a 12 ml 37° normothermic (n = 14) or 24° hypothermic (n = 12) aortic root injection of amiodarone (.25 mg) in .9% saline or .9% saline alone. The hypothermic group was allowed to rewarm spontaneously. Hearts were then subjected to 30 minutes of global ischemia within a water jacketed chamber at 37°C followed by 10 minutes of aortic re-perfusion with oxygenated buffer. Following 10 minutes in the working heart mode, hemodynamic determinations were remeasured during steady state conditions.

Amount of drug in mg in 12 ml 0.9% saline Injectate	N	Recovery	Aortic Flow Preischemic	Aortic Flow Postischemic	Percent Recovery
			$\bar{x} \pm S\bar{x}$		
0	13	4/13	47 ± 2	5 ± 3	10 † †
0.125	9	9/9	45 ± 2	16 ± 4	37 * * † †
0.250	10	10/10	42 ± 2	30 ± 2	70 * *
0.500	13	10/13	46 ± 1	18 ± 4	39 * †
3.0	14	10/14	47 ± 2	20 ± 5	42 * †
6.0	12	9/12	47 ± 2	16 ± 4	34 * †
12.0	7	7/7	46 ± 4	13 ± 3	28 * † †

* $p < 0.05$ and ** $p < 0.01$ vs controls
† $p < 0.05$ and †† $p < 0.01$ vs 0.25 mg dose

This screening study demonstrates a narrow dose response for amiodarone. Unknown is the effect when added to clinical cardioplegic solutions or under cold conditions. Small doses 125 µg/gm of wet heart weight appear to be protective against global ischemia.

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NORMOTHERMIC AND HYPOTHERMIC DATA

Groups	N	Survivors	Preischemic aortic output (mL/min)	Postischemic aortic output (mL/min)	Recovery of function
Normothermic control	8	2/8(25%)	41.4 ± 1	4.5 ± 4	12%
Normothermic amiodarone	6	6/6(100%)	41.7 ± 2	25.4 ± 2	61%
Hypothermic control	6	4/6(66%)	41.5 ± 2	15.8 ± 7	38%
Hypothermic amiodarone	6	6/6(100%)	43.0 ± 4	34.5 ± 4	81%

The unique characteristics of amiodarone as an established antiarrhythmic and possible calcium channel blocker may have significant implications toward potential use of the drug as a cardioplegic ingredient. The results suggest that amiodarone ameliorates the deleterious effects of global ischemia in hypertrophied rat hearts at normothermic and moderately hypothermic temperatures. The protective mechanism of amiodarone may be secondary to 1) calcium channel inhibitor, 2) increased collateral blood supply to myocardium, 3) antiarrhythmic potential or 4) vasodilatory effect of the drug.

Proposed Course: Studies at 28, 18 and 10°C with buffer and cardioplegic solutions will be performed. Oxygen consumption, positive and negative left ventricular dP/dt, and myocardial ATP levels will be assessed. If efficacy can be demonstrated, studies stimulating clinical conditions will be performed in sheep.

Publications:

Takach, T.J., Jones, M., and Clark, R.E.: Protective Effect of Amiodarone during Global Ischemia under Hypothermic and Normothermic Conditions in Hypertrophied Hearts. Surgical Forum XXXV: 341-344, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02749- 02 SU

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one lina between the borders.)*
Development of angioscopy for visualization of coronary arteries

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, leboratory, and institute affiliation)*

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Martin Leon, M.D., Senior Investigator, Cardiology Branch, NHLBI
Paul D. Smith, Ph.D., Senior Investigator, BEIB
Howard S. Kruth, M.D., Senior Investigator, Lab of Experimental Atherosclerosis
Jim Voigtlander, Technician, Surgery Branch, NHLBI
Rufus C. Seabron, Technician, Lab of Experimental Atherosclerosis, NHLBI
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COOPERATING UNITS *(if any)*
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Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The rationale for this investigation was the concept that visualization of intralumenal vessel pathology is essential to laser ablation of atheromatous obstructions. Various fiberoptic catheters were evaluated which ranged in size from 1.5 to 2.8 mm in diameter. A unique adapter system was developed which permitted large image video projection from the camera to the CRT. A series of flexible sheath systems were designed, fabricated and tested as was a variable infusion system. The complete arterial vasculature of dogs, sheep, swine and postmortem humans have been inspected and video recorded. The coronary circulation in sheep was visualized using cardiopulmonary bypass with aortic cannulation of the angioscope. Three human subjects have had studies of the proximal portions of both the left and right coronary arteries.

Sheep arteries, after angioscopy were perfusion fixed in silver chloride to study endothelial injury. The data demonstrate that in vivo visualization of the central and peripheral arteries and veins is feasible with minimal injury and no organ damage. New guidable systems with additional ports for manual extraction of intralumenal obstructions are in a design phase.

F45

Project Description: Various fiberoptic bronchoscopes modified for intravascular visualization and a aqueous environment were obtained from three manufacturers. Each was coupled to a miniature color TV camera by a lens coupling device developed in BEIB. Appropriate video monitors and tape recording systems were purchased. A variable rate perfusion system was designed and fabricated to permit intravascular visualization with clear media. Various materials were used to fabricate a vascular sheath through which the angioscope is guided into the blood vessel.

Studies were conducted on anesthetized swine and dogs to study the peripheral carotid and femoral arteries through incisions in the neck and groin areas. Coronary arteries were visualized in postmortem human hearts and arrested sheep hearts on cardiopulmonary bypass by inserting the angioscope into the aorta through a small incision controlled by suture. In human subjects, the proximal coronary arteries were visualized by direct cannulation during aortic valve surgery. These experiences have provided guidelines for improved design. These include tip flexibility distal and proximal balloons, a vacuum channel, and a channel of sufficient size to accommodate microforceps.

Proposed Course: Further development and evaluation of the NIH angioscopic system. Animals with atherosclerosis (rabbit and swine) will be used for in vivo assessment of safety and efficacy of new devices.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02750-02 SU

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of various laser sources on atherosclerotic coronary arteries

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Martin Leon, M.D., Senior Investigator, Cardiology Branch, NHLBI
Robert Bonner, Ph.D., Senior Investigator, BEIB
Paul D. Smith, Ph.D., Senior Investigator, BEIB
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI
William C. Roberts, M.D., Chief, Pathology Branch, NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI
John Deignan, Chief, Laser Laboratory, Goddard Space Flight Center, NASA

COOPERATING UNITS *(if any)*

BEIB
Cardiology Branch, NHLBI
Pathology Branch, NHLBI
NASA Goddard Space Flight Center
Naval Research Laboratory

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The optimum laser source for performing coronary artery laser angioplasty has not been determined. The purpose of this study is to define the most suitable laser source for vaporization of atherosclerotic plaque. Selected coronary arteries from human cadavers are exposed to lasers of various wave lengths and precise real time measurements of thermal diffusion are made by thermocouples and infra-red photography.

In addition, well defined gross and histopathological techniques are used to compare the effects of various laser-tissue interactions. Four laser sources have been tested. Argon creates a large thermal injury regardless of pulse duration, number of pulses and energy per pulse. Carbon dioxide caused thermal injury to human arteries similar to argon but this can be minimized by alteration of the three major variables. Nd-Yag systems are suitable but require alteration of the natural wave length. The most efficacious laser sources were xenon chloride and krypton fluoride. These lasers can be highly controlled and vaporize atheroma well with minimal thermal effects. These studies provided a basis for acquisition of an excimer laser.

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Project Description: The coronary arteries from twenty patients with atherosclerotic coronary vascular disease are obtained shortly after death. These specimens were then used to test various laser sources in time-energy studies.

Using fast reactive temperature thermocouples on the adventitial surface near the target temperature diffusion gradients were calculated for each wave length at different energy exposures. Simultaneously, an infrared thermal camera was positioned to record the surface temperature at the laser-tissue interface.

After several exposures using differing energy levels for each laser wave length these coronary artery specimens were processed for gross and histological evaluation.

In these ways a profile for each laser wave length was developed enabling us to decide which wave lengths maximized laser-tissue interaction but minimized thermal diffusion to surrounding normal tissue (arterial wall).

Pulsed carbon dioxide caused variable tissue effects. Very short pulse durations (< 10 msec) at constantly energy resulted in a decrease in thermal injury. Argon, regardless of variation of pulse duration, frequency and power levels caused the greatest amount of thermal injury. Xenon chloride and Krypton fluoride were highly controllable and produced razor cut-like lesions without thermal injury. On the basis of these studies, an excimer laser was purchased.

Proposed Course: This portion of the laser angioscopy project has been completed.

Publications:

Underhill, D.J., Smith, P.D., Leon, M.B., McDonald, J.R., Bonner, R.F., Clark, R.E.: High resolution angioscopy: feasibility, limitations, and design considerations for laser coronary angioplasty. Surgical Forum, XXXVI, 1985 (in press).

ESP

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02752-02 SU

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Augmentation of vascular supply to ischemic myocardium

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jose Montalvo III, M.D., Clinical Associate, Surgery Branch, NHLBI
Joseph Dodd, M.D., Clinical Associate, Surgery Branch, NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI
Ellis Unger, M.D., Clinical Associate, Cardiology Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*
Cardiology Branch

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this study is to determine the feasibility of augmenting myocardial blood flow by extracardiac methods. Specifically, the use of the internal mammary implant coupled to laser drilled ventricular channels and omental and/or splenic onlay wraps are to be considered. Additionally, the use of endothelial cell seeding together with growth factor may provide further augmentation. The rationale of the proposal is that many patients are not candidates for the coronary artery bypass procedure because of extreme atherosclerosis. There is a possibility that one of the major deficits in long term IHSS patients is ventricular mass/vascular supply disproportion. As an initial step to determine feasibility and develop techniques, forty dogs, in various groups were treated with combinations of the internal mammary implant, omental wrapping, and application of ameroid constrictors (AC). These groups were (1) control of IMA implant which was ligated and an AC applied to the left anterior descending coronary artery (LAD); (2) same as group 1 but no IMA ligation; (3) same as (2) plus omental wrap after epicardial abrasion; and (4) same as (2) except additional AC applied to the circumflex artery. The results show that the IMA remained patent in all cases and collateralized flow to the LAD and circumflex arteries. No animals developed myocardial infarction except group(1) controls. Blood flow through IMA averaged 8 ml/min which increased 33-50% with catecholamine stimulation. Microsphere studies demonstrated an augmentation of 0.15 ml/gm of heart at rest and a 33% increase with stimulation. No influence of omental wrapping on flow augmentation was shown. The data show that augmentation is possible with internal mammary artery implantation in the ischemic myocardium.

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Project Description: Large dogs, 125-130 kg with adequate sized internal mammary arteries were used. A left thoracotomy was made and dissection of the left internal mammary artery (IMA) was performed. The heart was exposed, the IMA ligated and transected distally with at least two open side branches. The IMA was tunnelled into the mid myocardium and suture fixed in place. An ameroid constrictor was placed on the anterior descending artery above the first septal perforating vessel. Constriction was obtained gradually over a 54 day interval. Additional ameroids were placed on the circumflex artery to provide sufficient ischemic stimulus for the development of IMA communication to the sinusoidal system. Serial studies included contrast and nuclear angiography, hemodynamics, and microsphere determination of collateral blood augmentation. Extensive histologic, dye fixation and radiographic studies were performed. Additional operative techniques used free omental grafts obtained by laporotomy. Data for IMA flow and blood flow distribution are shown below:

IMA FLOW

	Q-ml/min-rest	Q-stimulated	HR rest	HR stimulated
IMA-LAD-AC	9.7	12.3	92	133
IMA+omentum + LAD-AC	6.4	8.8	79	127
IMA-LAD+ Circ-AC	12.0	25	60	110

BLOOD FLOW DISTRIBUTION - ANTERIOR WALL

	Baseline	Occlusion	Pace	Pace + Occlusion
IMA-LAD-lig	0.38	--	0.59	--
IMA-LAD-AC	0.60	0.47	0.80	0.62
IMA+omentum + LAD-AC	0.53	0.38	0.78	0.65
IMA-LAD+ Circ-AC	0.32	0.29	0.41	0.20

Posterior wall flows ranged from 0.4 to 0.75 ml/gm at rest and were statistically greater than anterior wall flow in all groups except the last.

These data demonstrate that IMA implants can collateralize ischemic segments of myocardium and flow can be increased by catecholamine stimulation. Double implants with two ameroid constrictor placement are now in progress.

Other approaches to augment the vascular supply to the ischemic myocardium include CO_2 and excimer drilling of the left ventricle. Excised sheep hearts have had perforations created by CO_2. The beam width is not uniform at present and an articulated arm and mirror collometer has been purchased to solve this problem.

The excimer studies await the startup of this new system. If small, multiple perforation of uniform size can be obtained to mid myocardium by either CO_2 or excimer lasers, further augmentation will be attempted using vascularized pedicles of spleen or omentum in conjunction with endothelial seeding and growth factor infusions.

Proposed Course: Continuation of this project with sequential steps as outlined.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02753-02 SU

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development evaluation of a synthetic trileaflet valve

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Victor Ferrans, M.D., Ph.D., Chief, Ultrastructure Sect, Pathology Branch, NHLBI
Ram Paul, B.S., Medical Technician, Technical Development Lab, NHLBI
Joseph E. Pierce, DVM, Chief, Section on Laboratory Animal Medicine & Surgery, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*
Pathology Branch, NHLBI
Technical Development Laboratory, NHLBI

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

During a 13 year program, a synthetic trileaflet valve has been developed. Prototype clinical valves use a narrow soft sewing collar of knitted polyster, a flexible coronal shaped stent and a micro-woven fabric which is highly flexible and has stiffness and anisotropic properties similar to normal aortic leaflet tissue. Extensive durability and soft tissue implant studies were performed prior to mitral valve replacement in sheep. The in vivo data showed that valvular insufficiency occurred early (24 hours) which has subsequently been traced to alteration of filament geometry from stress compaction. New fabric designs have been completed and a second prototype is in production. Valves are inserted into a hydraulic testing device which characterizes forward and regurgitant power losses. Accelerated fatigue testing is performed at cyclic rates of 1000-1200/min. Implant studies consist of subcutaneous placement of materials in rabbits, conduit insertions in the arterial and venous systems of dogs and baboons and valve insertion in juvenile sheep.

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Project Description: The purpose of this investigation is to develop a durable trileaflet prosthetic aortic valve made entirely of synthetic materials. The design, materials and compounds with the base materials provide thrombo resistant surfaces and initial antimicrobid properties. Total power loss is approximately 4%. Durability in vitro ranges from 600-800 million cycles. The fabric leaflets have had extensive in vivo implant testing demonstrating no long-term biogradation. Filament size, number, fabric construction, and pore size and spacing are major variables. New construction of small pore (15-35 mm) fabrics has used copolymers of polyesters.

Mitral valve replacement is performed in sheep and periodic assessments are made by cardiac catheterization, angiographic, and ultrasonic studies. Post-mortem analyses are performed which include macro and microscopic photography, electron microscopy, and atomic absorption spectral data for mineralization. Twelve implants have shown similar patterns of mild to severe regurgitation and two which were collagen coated calcified within twenty weeks.

Proposed Course: New fabrics are under construction. An altered leaflet geometry design should provide greater coaptation surface area and prevent regurgitation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02754-02 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Blalock-Taussig shunts with prosthetic grafts: long-term observation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Jose Montalvo, III, M.D., Clinical Associate, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The hypothesis tested in these studies was that prosthetic grafts of specific diameters, biomaterial composition, and lumenal topography have different long term patency rates when used as brachiocephalic to pulmonary artery conduits. The specific aims were to test various types of grafts made from knitted and woven polyster (with and without internal/external velour), microporous expanded polytetrafluorethylene (EPTFE), bovine pericardium or ubilical vein. Fifty-four rhesus monkeys had a left thoracotomy and various grafts (4, 5, and 6 mm) with lengths of 2-5 cm inserted between the systemic and pulmonary circulations. There were eight deaths prior to 3 month catheterization. One monkey was unable to be catheterized secondary to poor vessels. Thirty of 45 grafts were patent at 3 months and 23 of 25 patent at 12 months. EPTFE showed a similar patency rate of 85% at 3 months and 12 months in 6 mm diameter prosthesis and 60% in the 4 mm size. Other materials were less satisfactory. These data suggest that when prosthetic conduits are used for palliation of pulmonary oligemia in infants and young children, the choice of conduit is highly important for long-term palliation. Further, prosthetic materials do not provide the long lasting palliation of the native subclavian artery as described in 1948 by Blalock and Taussig. Of the prosthetic materials available, EPTFE has the most consistent patency rates in 4 and 6 mm sizes. Patency rate is influenced by prosthesis diameter.

PSY

Project Description: Mature Rhesus monkeys of either sex had a left thoracotomy in the third intercostal space. Four, 5, or 6 mm prosthetic grafts varying in length from 2-5 cm were anastomosed to the subclavian artery or aorta and the left pulmonary artery. Changes in pulmonary and aortic blood flow and pressures were measured. Cardiac catheterization was performed at 3 and 12 months after operation. At 12 months, sacrifice with hemodynamic measurements through the open chest was performed. Conduit gradients, shunt flows and pressures were determined. The prostheses were submitted for histologic examination. Graft occlusion which occurred early initiated at the proximal and/or distal anastomosis. Few long-term patent grafts had complete endotheliazation and most had anastomotic intimal hyperplasia. The patency rates are shown below.

	3 Month Patency	12 Month Patency
6 mm Pericardium	100%	87%
6 mm Gortex	85%	83%
4 mm Pericardium	0	0
4 mm Umbilical Vein	71%	40%
4 mm Gortex	66%	66%
5 mm Woven Dacron	0	0
5 mm Knitted Dacron	100%	66%

Proposed Course: The study has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02758- 02 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Creation of accelerated atherosclerotic lesions in swine

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

David J. Underhill, M.D., Clinical Associate, Surgery Branch, NHLBI

Martin Leon, M.D., Senior Investigator, Cardiology Branch, NHLBI
Joseph E. Pierce, D.V.M., Chief, Section on Laboratory Animal Medicine & Surgery
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI

COOPERATING UNITS *(if any)*

Cardiology Branch, NHLBI

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

As part of a project to perform successful laser angioplasty, it is necessary to develop a swine colony with severe atherosclerotic vascular lesions within six months. The acceleration is obtained by balloon endothelial denudation of carotid and femoral arteries after which the animals are placed on a cholesterol-laden, high fat diet.

Development of vascular lesions will be assessed by ultrasonic, nuclear, and angiographic methods. Animals with severe lesions will be used for the angioscopy and laser angioplasty studies.

856

Project Description: We have secured a colony of 20 Hormel Miniature swine. They have been placed on a 2% cholesterol diet for one month prior to undergoing endothelial balloon denudation with a Fogarty catheter. Fourteen swine have been denuded to date and included the carotid arteries and ilio-femoral vessels. The remaining six swine will be done in the near future. They will then be kept on this "atherogenic diet" for six months with serial cholesterol level measurements and intermittent angiography of selected animals.

Proposed Course: This colony is being raised in order to provide a suitable animal model to study the *in vivo* effects of laser-tissue interaction and furthermore as a live model on which to perform laser angioplasty (Please see the review on Laser vaporization of atherosclerotic plaque in swine.)

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02761-01 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The use of alpha adrenergic blockade as a myocardial protective agent

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Lawrence R. Glassman, M.D., Clinical Associate, Surgery Branch, NHLBI
Thomas J. Takach, M.D., Clinical Associate, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The utility of phentolamine, an alpha adrenergic blocking agent, as a myocardial protective agent is being studied. The model for study is one of global ischemia and reperfusion. The animal for study is the genetic strain of spontaneously hypertensive rats. The preparation is a combination of the original isolated heart apparatus as defined by Langendorff and the working left atrial perfusion modification of Neely. The data generated at this point have shown that phentolamine ameliorates the deleterious effects of global ischemia. This effect was observed at 38°C and at 25°C. Additionally, salutory effects were observed when the drug was given at the onset of ischemia or at the initiation of reperfusion.

454

Project Description: Spontaneously hypertensive rats were studied as an isolated heart preparation. After anesthesia the hearts were rapidly removed from the animals via a combination abdominal and trans-diaphragmatic approach. The hearts were arrested in iced buffer solution. The aorta was attached to the perfusion system and the hearts were studied for pre-ischemic hemodynamics. Aortic output, aortic blood pressure, heart rate, and coronary sinus flow were measured. The hearts were then made ischemic at either 38 or 25°C. The hearts were injected with one of a series of six drug dosage schedules. This provided a control and two experimental groups at each temperature. After the ischemic interval of 30 minutes at 38°C and 90 minutes at 25°C, the hearts were reperfused. Post ischemic hemodynamics were measured. The data show a significant difference in all groups treated with phentolamine when compared with the appropriate control groups.

Proposed Course: Studies with the isolated rat heart preparation will continue at temperatures of 4-15°C and then be extended to an *in vivo* sheep preparation.

Publications: Glassman, L., Takach, T., Weber, S., and Clark, R.: Inhibition of alpha adrenergic receptors with phentolamine provides myocardial protection during global ischemia. Surgical Forum XXXVI (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02762- 01 SU

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chronic mitral insufficiency

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Robert J. March, M.D., Clinical Associate, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This laboratory study tests the hypothesis that left ventricular function following mitral valve replacement may be better preserved by retaining the tethering effect of the mitral valve apparatus in chronic mitral regurgitation.

The specific aims are as follows: we will develop a chronic animal model of mitral regurgitation which allows the repeated measurement of left ventricular systolic and diastolic dimensions and mechanics, regurgitant and forward flow across the valve (directly and via Doppler ultrasound), myocardial oxygen consumption, and hemodynamics. The effect of the acute loss of all the mitral valve attachments versus loss of the anterior leaflet attachments versus preservation of all structures on left ventricular mechanics, dimensions, and metabolism will be determined. The utilization of a specially designed left atrial flow transducer allows direct measurement of regurgitant flow thus permitting further validation of color Doppler mapping - a technique being investigated in this laboratory.

f60

Project Description: Utilizing lambs, obtained from a mixed breed of Ramboulet and Dorset sheep with weights of 20-25 kg, we have created 4.5 mm circular defects in the anterior mitral leaflet through a left atriotomy while on cardiopulmonary bypass and under sterile conditions. A left intra-atrial, supra-anular, electromagnetic flow transducer is sutured into place and another transducer is placed around the aorta. Six millimeter piezoelectric crystals are affixed to the epicardium to monitor left ventricular dimensions. Initial hemodynamic data is recorded and Doppler studies completed. The animal is allowed to survive 2-3 months after which reoperation and mitral valve replacement is performed. After a short period of intraoperative recovery, the animals will undergo transventricular detachment of portions of the mitral apparatus with fine wires. Left ventricular dimensional and functional changes will be measured under controlled conditions.

The acute loss of mitral valvular attachments following valve replacement in a chronic model has not yet been studied by other investigators. This study should provide insight into the pathophysiology of the left ventricular dysfunction following valve replacement in adult patients with chronic mitral regurgitation.

Proposed Course: Thus far, ten animal models have been created. Valve replacements will commence in the near future. The study is to continue.

FY 1

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02763-01 SU

PERIOD COVERED
October1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 chererters or less. Title must fit on one line between the borders.)*
Prostaglandin B-X - A new agent for myocardial preservation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

Lawrence R. Glassman, M.D., Clinical Associate, Surgery Branch, NHLBI
Thomas Devlin, Ph.D., Chairman, Department of Biologic Chemistry
Hahnemann Medical College, Philadelphia, PA
Richard E. Clark, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS *(if any)*
Hahnemann Medical College

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

Prostaglandin B-x is a short chain polymer of prostaglandin B. It is unique as a preservation agent in that it acts to protect mitochondria from the stress of an ischemic insult. In this polymerized form it appears not to possess any of the properties of the prostaglandin family from which it was derived. The drug is being tested as a potential agent for myocardial preservation on isolated hearts. The animal models being tested include both spontaneously hypertensive rats and Sprague-Dawley non-hypertensive rats. The preliminary data show activity of PBx at 38°C in dosages of 80-120 mg per heart which resulted in a 50-70% functional recovery of the left ventricle after 40 minutes of ischemia. No controls (7) had any measurable recovery with this duration of ischemia.

f62

Project Description: Both Sprague-Dawley and spontaneously hypertensive rats are being studied in an isolated working heart preparation. After anesthesia the hearts are removed and then arrested in an iced buffer solution. The hearts are attached to a perfusion apparatus via the aorta and the pulmonary veins. Pre and post ischemic hemodynamic measurements are made. The control animals are given a placebo solution while the experimental groups are given varying doses of prostaglandin Bx by infusion into the aortic root after the onset of ischemia. Dose response relations for each temperature used (38, 25, 15 and 5°C) in each of three solutions (saline, Krebs-Henseleit, and a cardioplegic solution) with and without high oxygen tensions will be developed.

Results: Concentrations of 3.3 - 66.7 µg/ml were tested in normal saline with 40 minutes of global ischemia at 38°C. Doses in the range of 6-6-10 µg/ml provided 50-70% recovery of left ventricular function. These dosages correspond to 80-120 mg per heart or approximately 60-100 µg/gm wet heart weight.

Proposed Course: To continue protocol.

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

OCTOBER 1, 1984 TO SEPTEMBER 30, 1985

Separation Science Instrumentation

We have continued to expand the capability of high-speed countercurrent chromatography (CCC), the most advanced form of CCC recently developed in our laboratory. Our efforts were directed toward three goals:

1. Development of large-scale preparative CCC. 2. Improvement of the table top multi-layer coil planet centrifuge in partition efficiency, speed and versatility for separation of polar compounds. 3. Development of a novel foam separation method which is based on the dual countercurrent system.

Development of a large-scale preparative CCC scheme was focused on the hydrodynamic studies on a two-phase solvent system composed of n-butanol/acetic acid/water (4:1:5) which is most commonly used in separation of biologically active polar compounds. The overall results indicated that both retention of the stationary phase and peak resolution of peptide samples can be optimized at a relatively low revolutional speed of 300 rpm where 1 gram quantity of the material was well separated with a 750 ml capacity column in a few hours. The table top model of the coil planet centrifuge for high-speed CCC was improved by adding a temperature-control system to allow the efficient use of hydrophilic solvent systems for separation of polar compounds. Capability of the apparatus was demonstrated on one-step purification of two crude synthetic peptides at 50°C which yielded 90% - 98% purity fractions in short times ranging from 30 minutes to 2 hours. For the rapid and efficient separation of macromolecules and cell particulates, a new foam separation method was devised. The method uses a coiled tube equipped with 5 flow channels, two at each end and one sample feed line at the middle portion of the coil. Surfactant solution and nitrogen gas are simultaneously introduced into the opposite ends of the rotating coil to generate foam. The sample mixture introduced at the middle portion of the coil is continuously separated according to the foam affinity: any material having the affinity to the foam is quickly carried with the foaming stream and eluted out from the tail of the coil while other materials are carried with the liquid stream in the opposite direction and eluted out through the head end of the coil. Efficiency and versatility of the present method were fully demonstrated on separation of a dye mixture with sodium dodecyl sulfate as a collector. Successful results of preliminary protein separation, bovine serum albumin and sheep hemoglobin, strongly suggest that the method may be efficiently applied for affinity separation of biologically active materials, including macromolecules and cell particulates.

Electron Spin Resonance Development for Medical and Biological Problems

The goal of this project is to develop and adapt electron spin resonance spectroscopy to study the biochemistry, physiology, and pathology of cells and tissues in order to answer problems of medical and biological importance. To accomplish this goal we are working on approaches to increase the sensitivity and developing cavity design suitable for different problems ranging from microsamples, to cultured cells to whole tissues.

By utilizing different frequency microwave sources it is possible to optimize resonator design for each type of biological sample. Initially we assembled an X-band, 9 GHz spectrometer. This spectrometer is useful for conventional chemical or biochemical measurements but is limited to the study of small aqueous samples of 1-4 mm in diameter. Various resonators were designed and tested with the X-band spectrometer system including several loop gap resonators. In order to accommodate large aqueous samples such as living perfused organs work was started on development of L band, 1-2 GHz, and S band, 2-4 GHz, spectrometer systems. An S band loop gap resonator was designed and built to enable the study of free radical generation in living perfused hearts. This is of great importance since free radical generation is thought to occur in the post ischemic heart and to mediate ischemic and reperfusion cell damage.

Over the past year we have focused on 2 important cardiovascular applications (1) the mechanism of the adriamycin cardiomyopathy (2) the mechanism of ischemic and post ischemic heart damage. Adriamycin is one of the most potent anticancer drugs in clinical use but it is also very toxic to the heart and induces a cardiomyopathy which is the third most common cardiomyopathy in the United States. Numerous studies have suggested that the therapeutic and toxic effects of adriamycin are due to the formation of reactive oxygen and drug radicals but the mechanism of radical formation was unknown. Using ESR we demonstrated that Fe(III) binds to adriamycin and that these complexes cycle to reduce oxygen. Adriamycin reduces its bound Fe(III) to Fe(II) with the formation of an oxidized adriamycin free radical. The Fe(II) then donates an electron to molecular oxygen forming superoxide and hydrogen peroxide. This mechanism explains the formation of reduced oxygen and drug radicals which are thought to mediate adriamycin's therapeutic and toxic effects.

Free radicals are thought to be generated in the ischemic and reperfused heart and to mediate the cellular damage which occurs. We developed an ESR technique to directly measure free radical generation in the ischemic and post ischemic heart. These studies demonstrate marked free radical generation in the post ischemic heart and provide the first direct evidence for free radical generation in the post ischemic heart.

Biophysical Instrumentation

The flow apparatus we have been developing is receiving considerable acceptance by the scientific and industrial community. Over two hundred

different groups are now using the "Berger Ball Mixer" for both quench flow and stopped flow experiments. Because the quench flow instrument developed in this section is used so extensively for studies of ion transport by Sacroplasmic reticulum and ATPase, but uses a large volume of sample, we have developed a new drive for this instrument in which an inertial system transfers the momentum of a heavy fly wheel through an electric clutch to a cam that drives one to three syringes to constant velocity in less than two milliseconds. In the quench flow apparatus this means only 0.5 ml of scarce enzymes are needed per measurement instead of the present 2ml. Testing has been carried out on the quench flow apparatus to ascertain the minimum reaction time that can be observed. This appears to be at least as good as our existing quench flow apparatus and with some simple modifications will greatly increase the time resolution.

The use of microcalorimetry in biology has been relatively small over the years due to the size and concentration of the sample needed, the sensitivity of the instrument, the many artifacts associated with thermal reactions, and the long time need for sample equilibration. Often only two or three reactions can be performed per day. A new flow microcalorimeter has been developed in this section which uses only 50 µl of each reagent and can detect one nanomole of many reactions. The dissociation of NADASE Dimer to monomers has recently been studied with this machine. The concentration of Dimer in the 50 microliter samples used was 2.5×10^{-9} moles. The dissociation heat was about 20Kcal/mole. This gave 50 microcalories. At this point in the instrument development we have a sensitivity of .1 microcalorie but small artifacts raise the variation to 1 or 2 microcalories. One hour is still needed for the initial equilibrium to be reached but reactions can be run every 15 minutes after that assuming they are complete in that time. tRNA-tRNA transferase reactions as well as ACTase reactions are planned in the coming months. Experiments with scientists in NIADKK on the assembly of protein molecules to form clathrin indicated a reasonable amount of heat per bond assuming all amino acids are involved in the number of bonds formed. The overall heat per clathrin molecule formed i.e. 32 triskelions, was 10^6 calories.

Pulmonary and Cardiac Assist Devices

The Section on Pulmonary and Cardiac Assist Devices applies advances in technology and pathophysiology to assist the failing lungs and the failing heart. Such effort in technology includes the design and fabrication of blood access devices, the fabrication of pumps, gas exchange devices, and circulatory assist devices.

During the past year we have demonstrated the deleterious effects of mechanical pulmonary ventilators on healthy lungs of experimental animals under certain conditions. Healthy, sedated and paralyzed sheep were continuously ventilated to peak intratracheal pressure of 30 x cm H_2O while pulmonary function was monitored. Following 2 days of ventilation, these lungs progressively showed a reduction in total static lung compliance (TSLC) functional residual capacity (FRC), abnormal arterial blood gases, and progressive opacification of lung fields. Such changes progressed further

when mechanical pulmonary ventilation was continued for another 2 days for a total of 4 days., when the animals developed a fullblown ARDS (Adult Respiratory Distress Syndrome). The addition of positive end expiratory pressure (PEEP) of 10 or 15 cm H_2O did not halt this deterioration, raising questions of the merit of PEEP clinically. These data are in accord with clinical reports showing no difference in ultimate outcome in patients ventilated with, or without the addition of PEEP.

In a similar group of sheep ventilated at a peak inspiratory pressure of 50 cm H_2O, severe lung impairment developed within 1-2 days. This resulted in severe ARDS, at which time ventilator management was changed to state of the art clinical practice. Such treatment was of no avail, and all animals (8) died of severe respiratory failure.

Using the above animal model, we have now performed a randomized study where on developing severe ARDS, one group of animals was treated with conventional mechanical pulmonary ventilation; and a bypass group, using an extracorporeal membrane artificial lung with continuous positive airway pressure (CPAP) of 10 cm H_2O, and mechanical ventilation discontinued. Evidence of lung recovery first became evident after 24 hours of bypass with almost hourly improvement in arterial blood gases during the following 24 hours. By the end of the 2nd day of bypass, the animals could tolerate breathing room air, and were taken off bypass. There remained, significant restrictive lung disease with poor compliance, attesting to the severity of the pulmonary insult. The arterial blood gases were normal while breathing room air, and on CPAP.

This study confirms the severity of lung injury from mechanical pulmonary ventilation under conditions commonly used in clinical practice. As an alternative to increasing the ventilation pressure to achieve adequate gas exchange, a low flow bypass membrane lung system is shown to be effective in reversing or preventing lung damage by mechanical ventilation.

Cell Measurements System

The porous bottom culture dishes (PBCDs) and related devices developed in this laboratory for the sterile measurement of electrophysiological parameters of cell layers are used in over 50 laboratories in the U.S.A. and abroad. Two companies are devoting considerable effort toward producing commercial versions of the PBCDs using cellulose ester membranes (Millipore Corp.) and plasma treated polycarbonate membranes (Becton-Dickinson Corp.). Many types of epithelial cell layers are grown on the PBCDs using a variety of membrane materials. Recently, endothelial cell layers have been grown here at NIH on our PBCDs with collagen membranes. These membranes permit good microscopic observation. A defined medium has been developed for use with the PBCDs to optimize a differentiated function (transepithelial sodium transport by A6 cells from Xenopus laevis kidney) rather than to maximize

growth. Since the PBCDs facilitate the study of differentiated function, a culture medium which optimizes this function has obvious advantages.

The major role played by Ca^{++} in the regulation of transport and other cellular processes has prompted us to improve methods of measuring microns in diameter to enter many cells without causing damage, making reliable readings extremely difficult. Electrodes of this size have walls so thin that they may be totally hydrated near the tip and become Na^+ and K^+ electrodes due to the monovalent metals contained in the glass. This conflict between Ca^{++}, Na^+, and K^+ sensitivities is believed to cause the poor performance often seen for electrodes smaller than 1 micron in diameter at Ca^{++} activities below 10^{-6} molal. We have therefore designed, constructed, and used unique anular burners for making micropipettes of fused quartz which is free of metals other than the silicon of the silicone dioxide. An improved silanization method is being developed for these pipettes so that the advantages of the quartz can be fully realized.

Luminescence Instrumentation

The binding of cupric, nickel, and zinc ions to tryptophan, tyrosine, and various di- and tri-peptides containing these amino acids has been studied by fluorescence quenching. The proton dissociation constants have been determined for the ligands by fluorescence titration. The association constants of the metal ions for the various ligands have been determined by curve fitting to the quenching data in the case of copper and nickel ions. Zinc ion complex association constants were obtained by competition experiments. This relatively novel method for measuring the strength of binding is relevant not only to the interaction of metal ions and peptides, but binding equilibria in general.

The strong interaction of melittin with bilayer phospholipid membranes was shown by the release of a fluorescent dye from liposomes. Only a small number of melittin molecules was needed to effect dye release, indicating that the peptide formed channels through the membrane. Calmodulin formed a complex with melittin, thus inhibiting the latter's ability to lyse liposomes. A sensitive assay for calmodulin has been developed based on this phenomenon. The calmodulin-melittin complex is disrupted in the presence of phospholipids. The kinetics of this process are being studied by fluorescence anisotropy and stopped-flow measurements.

A new, laser based, time-resolved fluorescence spectrophotometer has been assembled, tested and modified. A modelocked, doubled Nd:YAG laser synchronously pumps a cavity-dumped dye laser whose doubled output is tunable ultraviolet (ideal for proteins and many membrane probes). The configuration was converted to "T-format" to provide a unique capability for simultaneous collection of emission anisotropy and decays. Recent steps we've taken to computer control scanning (and sample/polarization alternation) provide the optimum instrument for collecting accurate fluorescence emission decay

f6f

surfaces (rather than just isolated curves). "Global analysis" of these high resolution data sets will provide the utmost detail in studies of fluorescence in biochemical systems. The present form of the instrument, though incomplete, is already finding details in protein fluorescence undisclosed by other instruments. The present system has been applied to problems in biochemistry involving the enzyme glutamine synthetase the interaction of melittin and calmodulin, bovine serum albumin, alcohol dehydrogenase, liposome-dye complexes, and excited state protonation of serotonin.

An atlas of fluorescence properties of about 30 protein dyes have been prepared and published. Various fluorescent or fluorogenic labels which react covalently with proteins have been attached to ovalbumin, and the excitation and emission spectra obtained. The spectra were then corrected for lamp and detector nonlinearities with wavelength. The atlas also contained fluorescence lifetime data on each dye-ovalbumin complex. This compendium of spectral and lifetime data is the only one presently available of its kind.

Clinical Devices

A micro machine for flame working quartz has been constructed and tested as a means of constructing a semi-automatic injector to be applied to insert DNA into the nuclei of human white cells. Preliminary results have demonstrated the feasbility of the idea but the very small size required has not been attained.

Collagen extension angiocatheters continue to be the most promising means of reaching through tortuous vessels with a minimum flow restriction but occasional rupture of the collagen sheath discourages human use. Some recent improvement in strength is expected to solve this problem. In addition the methods for making collagen catheters has been applied to construct collagen prosthetic blood vessels in the 3 mm size range that are being evaluated in the surgical research program.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01404-17 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Membrane Lung System for Long Term Respiratory Support

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	T. Kolobow	Medical Officer	LTD:NHLBI
	P. Prato	Visiting Fellow	LTD:NHLBI
	K. Tsuno	Guest Worker	LTD:NHLBI
	M. Borelli	Visiting Fellow	LTD:NHLBI
	R. Spatola	Guest Worker	LTD:NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

Section on Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4	4	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have performed long term studies on the effect of mechanical pulmonary ventilation at peak airway pressure of 50 and 30 cm H_2O in healthy adult sheep. Following some 24-72 hours of mechanical pulmonary ventilation at those respective peak airway pressures, the total static lung compliance, functional residual capacity, and arterial blood gases gradually deteriorated, leading to adult respiratory distress syndrome (ARDS). From such a point on, recovery with any form of mechanical pulmonary ventilation was no longer possible. However, a similar animal with ARDS when placed on extracorporeal membrane lung bypass and kept on CPAP of 10 cm H_2O had a rapid improvement in lung function, great improvement in arterial blood gases, and could be weaned off bypass with long term survival. We conclude that the mechanical pulmonary ventilator at high airway pressures is a major factor in the lack of healing of acutely diseased lungs. The membrane artificial lung can provide for oxygen delivery and CO_2 removal without the need for further mechanical pulmonary ventilation; such a course allows for full recovery in diseased lungs.

870

Objectives:

(1) The most widely used system for extracorporeal blood gas exchange is based on the bubble oxygenator system, first used at the advent of heart surgery. Systems based on the bubble oxygenator have now been available in disposable form for over 15 years. The advent of the disposable bubble oxygenator eclipsed most efforts at devising a safe perfusion system based on the admittedly superior performance of the membrane artificial lung.

While a perfusion system based on the bubble oxygenator is reasonably safe in short term applications, such device may become the limiting factor should the need arise to extend bypass for either technical reasons or otherwise. Moreover, for prolonged extracorporeal use such as cardiac assistance, or for respiratory assistance, the membrane oxygenator becomes a necessity. Systems based on the membrane artificial lung have been capable of safe operation for respiratory assist for up to one month. Hence, the membrane lung is ideally suited for the treatment of acute and chronic pulmonary insufficiency.

(2) To devise a safe extracorporeal perfusion system for extracorporeal removal of carbon dioxide in the controlof alveolar ventilation, and the control of breathing.

The natural lungs perform oxygen uptake and carbon dioxide elimination equally well. Similarly, both respiratory gases can be equally well removed through an extracorporeal membrane lung. Great practical importance lies in the observation that total metabolically produced CO_2 can be continuously removed by an artificial lung at blood flows only 1/2 to 1/5 of total cardiac output. (In contrast, oxygen transport requires blood flows equal to cardiac output). It therefore becomes possible to design a small, compact extracorporeal (or intracorporeal) membrane artificial lung for the removal of any desired fractionof metabolically produced CO_2, the purpose of which is to unload the natural lungs from the task of providing for CO_2 elimination.

Extracorporeal CO_2 removal is desirable in the control of breathing, and in patients on mechanical pulmonary ventilation in whom peak airway pressure is dangerously elevated. Through the use of such a system, the patient can immediately be taken off mechanical pulmonary ventilation. The projected patient population includes any patients with parenchymal lung involvement such as ARDS, neurogenic lung disease, hyaline membrane disease (HMD), meconium aspiration syndrome (MAS), bronchopulmonary dysplasia (BPD), the pump lung syndrome following total cardiopulmonary bypass using the bubble oxygenator system, following some types of drug ingestion-overdose, and other states of acute respiratory failure.

There is no scientific proof that an extracorporeal membrane lung perfusion system cen be of assistance in patients with chronic lung disease, particularly in those with significant CO_2 retention, and especially in

those already dependent on supplemental nasal oxygen inhalation. Such state of matters, however, does not preclude the possibility that extracorporeal CO_2 removal through a membrane lung may not impart some long lasting benefits to that patient population. This approach may "decelerate" the relentless deterioration so commonly seen in those patients with terminal chronic lung disease.

(3) Adult respiratory distress syndrome (ARDS)

(a) This syndrome at present carries a mortality of 90% in spite of best state of the art medical treatment. Of more serious concern is finding a patient on a mechanical ventilator after 24 hours, and inspiring 50% oxygen to have a survival rate of less than 35%.

The underlying cause of respiratory failure may be due to a variety of common, and not so common causes - including biological, physical, or chemical factors. The ultimate results are noncompliant stiff, difficult to ventilate lungs. Hence, it was told that the mechanical ventilator might provide the strength and the assistance to help the patient to ventilate its lungs. Unfortunately, a mechanical solution to pulmonary ventilation may not produce the desired effect without potential harm to those ventilated lungs. Damage from mechanical pulmonary ventilation, termed barotrauma, is real, and at times the direct cause for the patient°s ultimate demise from uncontrollable air leaks, secondary infection, multiorgan impairment including those of the renal, hepatic, and cardiovascular systems.

Management of patients with ARDS must be attuned to treatment modalities that will lead to recovery from respiratory failure as the end result. Transient "improvements" in arterial blood gasses do not attest to the correctness of the treatment, as such a course could inadvertently reduce the chance for later lung recovery.

Any treatment with a mechanical pulmonary ventilator must be proven in the laboratory in experimental animals. The end result of any treatment protocol must lead to full recovery. On the other hand, such questions become moot if pulmonary ventilation is dispensed with altogether, and to instead keep the patient on CPAP (continuous positive airway pressure), and on an extracorporeal membrane lung perfusion system.

(4) High pressure ventilation with a mechanical ventilator as the cause of lung disease.

(a) The mechanical pulmonary ventilator directs gas flow to the most compliant areas of the lungs, and largely bypasses the most diseased areas of the lungs. Thus, the remaining still normal areas of the lungs are continuously exposed to the excessive airway pressures and volumes. The long and short term effects of such ventilation have previously been ignored and overlooked.

In studies we reported previously, we have shown that healthy lungs ventilated at positive airway pressures of 50 cm H_2O developed serious lung impairment within 48 hours. That impairment leads to ARDS, with no recovery inspite of state of the art pulmonary management at a later time. We concluded that prolonged mechanical pulmonary ventilation at peak airway pressures of 50 cm H_2O and higher was inherently unsafe.

We have since shown in the same sheep animal model that peak airway pressures of 30 cm H_2O are no less dangerous if so maintained for 2-4 days, irrespective of respiratory rate, or PEEP. This finding was most surprising and disappointing, as many patients are often found at those ventilator settings. This finding, however, explains why the great majority of patients on 50% oxygen and on a mechanical ventilator for 24 hours or longer eventually died (ECMO study).

Methods Employed

(1) Blood access

The single catheter perfusion system developed in this laboratory has proven to be faultless in laboratory studies over the past 3 years. Blood flows have always been sufficient for the removal of continuously produced metabolic CO_2, and the supply of some oxygen. The advantage of such a method is in its immediate access to blood once the single catheter is introduced in the vein, the reduced number of vessel cannulation with concomitant reduction in possible bleeding sites, and the prepulmonary location of the perfusion system which returns to the lungs well oxygenated blood. Similar systems based on this design have performed well at other institutions.

I believe such single catheter based perfusion system is likely to become the method of choice in early stages of ARDS, so as to remove the patient from mechanical pulmonary ventilation, or to avoid placing them on mechanical ventilators.

(2) Lung injury from moderate pressure mechanical pulmonary ventilation

This study is a continuation of work begun last year. We have exposed sedated, paralyzed sheep to mechanical pulmonary ventilation at peak airways pressures of 30 cm H_2O. This pressure range is commonly seen in a hospital setting following surgical procedures, for example. We had expected to find little, or insignificant changes in lung function after 2-4 days of such mechanical pulmonary ventilation. Instead, in whatever combination we chose to study either low respiratory rate or high respiratory rate, with PEEP of 0, 10, 15, or higher, we invariably found severe deterioration in lung function following mechanical ventilation for 2-4 days. The extent of lung involvement was often substantially more affected than what could be visualized on standard chest x-ray films.

We have since continued our studies also at 50 cm H_2O peak airway pressure in the same animal model. The evolution to severe ARDS occurred within 1-2 days. Such continued insult if extended to 2-3 produced a decrease in total static lung compliance (TSLC), with a fall in FRC (functional residual capacity) to but half normal. At those endpoints there was severe hypoxemia with CO_2 retention. Continued ventilation of those lungs with any state of the art pulmonary management was utterly impossible, and all animals died while still receiving "optimal" respiratory care.

Our findings suggest that mechanical pulmonary ventilation at high airway pressure is the likely most important single cause of lack of healing, and in the progression of lung involvement to ARDS in patients with minimal to slight respiratory insufficiency, and who are then placed on a mechanical pulmonary ventilator. Such progression of lung disease from use of a mechanical pulmonary ventilator is time and peak airway pressure related. Our understanding of mechanical pulmonary ventilation as an important element in the evolving disease process can be of immeasurable help in the search for safe clinical practice.

At the same time, this animal model of ARDS has provided us with an excellent model of experimental ARDS. This model, we feel, is realistic as it in fact occurs daily in any hospital. We believe this model to be superior to models used in the past when acute lung injury was almost instantly produced followed administration and/or lavage with lineolic acid, hydrochloric acid, water, or saline, or following administration of endotoxins, etc.

(3) We have adopted the ARDS model induced by mechanical pulmonary ventilation as the model best suited to explore new and promising means of pulmonary management

In a study now underway, we induce severe ARDS through mechanical pulmonary ventilation at 50 cm H_2O a peak airway pressure. Once criteria of severe ARDS are met, based on FRC, TSLC, and arterial blood gases, the animals are randomized to a control group, and a treatment group. The control group is managed using state of the art mechanical pulmonary ventilation until death; the treatment group is placed on single catheter vein to vein extracorporeal membrane lung bypass, and mechanical pulmonary ventilation is immediately terminated. Instead, animals are allowed to awaken, and kept on CPAP of 10 cm H_2O, on 40% oxygen. Such approach immediately stops the mechanical pulmonary ventilator from being a continuing offender in damaging the lungs and allows the healing process to take over. With the artificial lung, a large fraction of metabolically produced CO_2 is continuously removed extracorporeally, reducing the need for much of mechanical pulmonary ventilation. The sheep continue to breathe at a low respiratory rate and tidal volume without any exertion. Hence, there is no need for mechanical pulmonary assistance. Meanwhile, some oxygen is also transferred to blood, raising PO_2, and permitting a reduction in FI02.

After some 24 hours, the PO_2 begins to rise rapidly, so that inspired oxygen concentration can be reduced within the next 24 hours to room air. However, these lungs may still not be able by themselves to remove the total load of CO_2. Hence, weaning over the ensuing hours must be tailored to CO_2 removal. Such weaning ordinarily occurs over a 24 hour period, following which bypass can be terminated and the sheep kept on CPAP.

However, there still remains significant abnormalities in TSLC, FRC, with chest x-ray films grossly abnormal, and the animal still needing CPAP for several more days. This picture suggests a severe underlying disease process that only slowly will resolve over time. Nevertheless, the animal is on room air, and maintains excellent arterial blood gases.

In this protocol we have shown for the first time anywhere, that induced and lethal ARDS can be reversed through the use of an extracorporeal membrane lung. Further, that the major reason to past failures at managing patients with ARDS has been the continued insult through mechanical pulmonary ventilation. The continuing use of the mechanical pulmonary ventilator at high airway pressures was, we believe, the immediate cause of the ECMO study failure.

Significance to biomedical research and the program of the institute

This laboratory has previously reported on new technology and design for the construction of safe perfusion systems. Such systems include the fabrication of cannulas, tubes, blood contacting surfaces, pumps, membrane artificial lungs. We have demonstrated the safety of those devices in extended animal studies. Such technology, while useful and new, cannot be transferred to the bedside without extensive proof of safety and efficacy in the laboratory setting. Hence, we had to develop a new, credible animal model of acute respiratory failure, and then apply our new technology to treatment of respiratory failure. Our results have been outstanding. A disease previously considered to have a 90% mortality rate may likely see survival rise substantially. Those encouraging results may be cause to accept patients for extracorporeal bypass at an earlier point, before significant iatrogenic lung disease from mechanical pulmonary ventilation.

Our results to date confirm our past experience reported in preterm fetal lambs, not treatable with state of the art ventilator care. Through the use of our extracorporeal perfusion system, within 24 hours those lambs were on room air, with normal arterial blood gases. All animals had been kept on CPAP while on bypass, at low airway pressure of 15 cm H_2O.

Thus, emerging technology to develop new concepts and devices must go hand in hand with our understanding of pathophysiology of disease. For instance, we have shown a major advance in medical care (the mechanical pulmonary ventilator) as the cause of great agony and misery. Clearly, the mechanical pulmonary ventilator had not been adequately tested for long term use in severely diseased lungs.

Our laboratory research on control of breathing, the development of a safe "third lung", the elucidation of elements leading to lung failure with increased dead space, left to right shunting, and others, have all helped us to understand the interplay of various factors leading to acute lung failure. From such work we now propose a viable approach to managing those patients in a manner likely to lead to enhanced survival. It may come as a surprise in this age of technological progress, that such an approach is likely to be overall less expensive, and hence, more cost effective.

We will continue our studies using the animal model with severely damaged lungs and treating such disease with an extracorporeal membrane lung. Such studies will be made definitive so as to establish the rationale of its use in the treatment of acute respiratory failure in the neonate, child and adult. We will continue to develop advanced components to improve on the extracorporeal bypass system.

Work will begin on partial, total, or demand cardiopulmonary assistance systems. Such a system will be comparable in complexity (simplicity) to the extracorporeal respiratory assist device described above. It will be designed for emergency, or elective applications, and will cover the entire spectrum of cardiopulmonary assistance. Short of an implantable booster pump, or a total artificial heart, this system will outperform all other modes of heart assist.

Publications

1. Zwischenberger JB, Toomasian JM, Drake K, Andrews AF, Kolobow T, Bartlett RH: Total respiratory support with single canula veinovenous ECMO: double luman continuous flow vs single luman tidal flow. Trans Am Soc Artif Int Organs, 1985.

2. Gattinoni L, Pesenti A, Caspani ML, Pelizzola A, Mascheroni D, Marcolin R, Iapichino G, Langer M, Agostoni A, Kolobow T, Melrose DG and Damia G: The role of total static lung compliance in the management of severe ARDS unresponsive to conventional treatment. Intensive Care Med 10:121-126, 1984.

3. Kolobow T, Gattioni L, Moretti MP, Prato P, Mascheroni D, and Tsuno K: Mechanical pulmonary ventilation at high airway pressures: Is it safe? Int. J. Artificial Organs, Vol 7, No. 6, 315-316, 1984.

4. Kolobow T: Mechanical pulmonary ventilation - a source of acute lung injury, Special Lecture No. 1, Japan Resusitation Society, Vol. 3, 9-12, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HL 01407-22 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Luminescence Spectroscopy in Biomedical Research

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	R. F. Chen	Senior Investigator	LTD:NHLBI
	C. H. Scott	Biologist	LTD:NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The binding of cupric, nickel and zinc ions to tryptophan, tyrosine, and various di- and tri-peptides containing these amino acids have been studied by fluorescence quenching. The proton dissociation constants have been determined for the ligands by fluorescence titration. The association constants of the metal ions for the various ligands have been determined by curve fitting to the quenching data in the case of copper and nickel ions. Zinc ion complex association constants were obtained by competition experiments. This relatively novel method for measuirng the strength of binding is relevant not only to the interaction of metal ions and peptides, but binding equilibria in general.

The strong interaction of melittin with bilayer phospholipid membranes was shown by the release of a fluorescent dye from liposomes. Only a small number of melittin molecules were needed to effect dye release, indicating that the peptide formed channels through the membrane. Calmodulin formed a complex with melittin, thus inhibiting the latter's ability to lyse liposomes. A sensitive assay for calmodulin has been developed based on this phenomenon. The calmodulin-melittin complex is disrupted in the presence of phospholipid. The kinetics of this process are being studied by fluorescence anisotropy and stopped-flow measurements.

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Project Description

Objectives:

The purpose of the work is to apply sophisticated techniques of fluorescence spectroscopy to specific problems of interest in biomedical research. In doing so, it is hoped that knowledge will be advanced in specific areas of biochemistry and that new methodology can be demonstrated to be useful.

Methods Employed:

Fluorescence and absorption data were obtained with Aminco-Bowman and Cary spectrometers. Chemical reagents were obtained commercially, and no human or animal experimentation was involved. Data reductions were largely done in MLAB, a program run on the DCRT DEC-10 computer. Purity of reagents were established by standard thin layer chromatographic methods.

1. The binding of several transition metal ions to tryptophan, tyrosine, and several di- and tri-peptides containing these amino acids have been investigated using fluorescence quenching. The utlraviolet fluorescence of these ligands is quenched upon binding to cupric and nickel ions, and the degree of quenching is a measure of complex formation. Quenching data were used with a curve fitting program to determine the association constants for these cations with various ligands. In the systems studied, the metal ion is competing with protons for binding to the free amino group;

$$H^+ + L \rightleftharpoons HL^+$$
$$Cu^{++} + L \rightleftharpoons Cu(L)^{++}$$

Here, L and HL^+ are free and protonated ligand, only the former of which can bind to Cu^{++}. The association constant for the second reaction can be determined if the pK_a of the first equilibrium is known. The latter was measured by pH titration of the ligands. These data yielded association constants, expressed as -log k, of between 6.5 and 8.1 for cupric ion complexes of trp-trp, val-trp, gly-trp, ala-trp, val-trp, trp-gly, phe-trp and others. The constants for Ni^{++} were about 2 orders of magnitude weaker. Zn^{++} binding does not cause quenching, but competition experiments with both Cu^{++} and Zn^{++} yielded the zinc association constants. Where available in the literature, reported constants were in good agreement with our results. Since binding is an important aspect of most molecular interactions in biology, this relatively novel method of assessing binding strength has significance for studying such interactions in general, and for studying peptide interactions with metal ions in particular. The mechanism of fluorescence quenching was probably energy transfer in the case of some colored ions, and enhanced intersystem crossing to the triplet state with other ions. In regard to the establishment of the validity of the method, the data are essentially complete and ready to be written up.

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2. The interactions between melittin and liposomes and calmodulin and melittin have been studied. We had previously found that melittin, a 26 amino acid peptide of bee venom, caused lysis of liposomes filled with a dye, 6-carboxyfluorescein (6CF). In calculating the number of melittin molecules required to release dye from one liposome, we conclude that only 10-20 molecules, corresponding to 3-5 melittin tetramers cause total release of dye from egg lecithin liposomes in less than 30 minutes. These data support the idea that melittin forms channels through the bilayer membrane, since only a few molecules are needed. In contrast, physical emulsification of the liposome phospholipids would require stoichiometric amounts of melittin. Because of the small amount of peptide required for release of the fluorescent dye, the system constitutes the basis of a sensitive assay for melittin and related peptides such as wasp mastoparan. In addition, it is now known that calmodulin forms a very strong 1:1 complex with melittin. We have shown that calmodulin inhibits the lytic property of melittin, but that lysis eventually occurs. This finding has results in 1) an assay for calmodulin based on its inhibition of melittin lysis of 6CF liposomes. This fluorometric assay is rapid and sensitive, with a limit of detection of less than 1 microgram of calmodulin (2 picomoles). The assay is comparable in sensitivity to the method based on calmodulin activation of phospholiesterase activity, a method requiring enzyme and a radioactive substrate; 2) further study on the kinetics of the breakup of the calmodulin melittin complex as evidenced by the time-dependent recovery of melittin lytic activity. We have studied the calmodulin-melittin complex by time-resolved and steady-state polarization of fluorescence and will apply stopped-flow methods.

Proposed Course:

We are presently assembling the ion complex data and data on the esterase properties of serum albumin for publication. It is intended to continue studying the melittin-calmodulin-phospholipid interaction kinetics with our stopped-flow and anisotropy methods. There are several intrinsically interesting projects which will be especially suitable for study with the laser-based nonosecond fluorescence instrument when the new minicomputer arrives. Specifically, the kinetics of excited state protonation of serotonin and melatonin, the resolution of the fluorescence of the two tryptophans in the spectrum of bovine serum albumin, and the use of difference decay curves to isolate spectra of individual dyes attached to proteins.

Publications:

1. Chen, R. F., and Scott, C. H.: Global and nonglobal rotations in proteins detected by fluorescence polarization, in Advances in Luminescence Spectroscopy, ASTM STP 863, L. C. Cline Love and D. Eastwood, Eds., Am. Soc. for Testing and Materials, Philadelphia, 1985, pp 26-39.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01408-20 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Methods in Fluorescence Spectroscopy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	R. F. Chen	Senior Investigator	LTD:NHLBI
	C. H. Scott	Biologist	LTD:NHLBI
	J. R. Knutson	Sr. Staff Fellow	LTD:NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

NHLBI NIH, Bethesda, Maryland

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new Nd:YAG laser based single-photon fluorescence decay apparatus has been assembled and tested. The mode-locked Nd:YAG laser output is used to pump a dye laser whose doubled output is ideal for excitation of the ultraviolet fluorescence fluorescence of proteins. Coupled with an automated sample changer and a new minicomputer, the instrument will represent the state-of-the-art in following fluorescence and anisotropy decay, as well as time-resolved emission spectra. The present system has been applied to problems in biochemistry involving the enzyme glutamine synthetase, the interaction of melittin and calmodulin, bovine serum albumin, liposome-dye complexes, and excited state protonation of serotonin.

An atlas of fluorescence properties of about 30 protein dyes has been prepared and published. Various fluorescent or fluorogenic labels which react covalently with proteins have been attached to ovalbumin, and the excitation and emission spectra obtained. The spectra were then corrected for lamp and detector nonlinearities with wavelength. The atlas also contained fluorescence lifetime data on each dye-ovalbumin complex. The compendium of spectral and lifetime data the only one presently available of its kind.

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Project Description:

Objectives:

The aim of this project is to develop instrumentation and methods of fluorescence spectroscopy which can be applied to biomedical research. Aside from techniques, the project includes the establishment of standards, evaluations of reagents and analysis of the accuracy and precision of methods.

Methods Employed:

Fluorescence spectra were obtained with an Aminco-Bowman spectrofluorometer, and corrections for lamp and detector variations with wavelength were applied by computer after digitization of the spectra. Fluorescence lifetimes were obtained with an Ortec single photon nanosecond fluorometer equipped either with a flash lamp or the laser system described below. Commercially available reagents were used and no human or animal experiments were involved. The time-resolved fluorescence data were reduced either with a DEC MINC computer or by a time shared PDP-10 computer at the DCRT.

Major Findings:

1. A fluorescence decay instrument has been built and tested with Dr. J. Knutson. The sample is excited by a tunable, synchronously pumped Rhodamine 6G dye laser. The pumping laser is a series 3000 Spectra Physics Nd:YAG acoustooptically modelocked laser emitting pulses of 1.06 micron light at 1 Mhz repetition rate. This infrared light is doubled by a KDP crystal, and the 530 nm light synchronously pumps the dye laser, whose output is angle tuned and doubled again, giving picosecond pulses (about 13 ps halfwidth) in the 270-320 nm region, ideal for exciting proteins. The high repetition rate and peak power, and the short pulse width, represent a great improvement over spark lamp sources. Single photon counting experiments are done in about 1/50 the time, and with greater precision. In addition to the laser, an automated cell compartment from PRA, Ltd. has been acquired and tested. It will be interfaced with a new Hewlett-Packard computer which is on order; this will allow automatic sample changing for blank correction, anisotropy data, and recording of time-resolved spectra. Much of the preliminary testing of the system was done with the DEC MINC computer with either PRA software or programs developed at Johns Hopkins University. The more advanced computer will be able to obtain time-resolved spectral and anisotropy data reduction utilizing "global analysis", a technique which requires high computational power to analyze several curves simultaneously. Lifetime and anisotropy data have already been accumulated on bovine serum albumin, glutamine synthetase, liposome-dye complexes, melittin and its complex with calmodulin, and a number of other systems. Some of these results have already been used by Dr. Ann Ginsburg (working with glutamine synthetase) and other results were presented at the meeting of the American Society for Photobiology, June 1985, in New Orleans.

2. Since the last report, we have completed and published an atlas of fluorescence spectra and lifetimes of various dyes attached to a protein, ovalbumin. This compendium consisted of about 30 dyes which were reacted with ovalbumin under identical conditions. The labeled protein was freed from unreacted dye by Sephadex chromatography. The spectra were taken at room temperature in pH 7.4 phosphate buffer. The data were corrected for lamp output and detector response using the MLAB program, which also did the drawings. Excitation and emission spectra for all complexes were presented and data summarized in a table. Representative fluorescence decay curves were included. The decay data were obtained with an ORTEC fluorescence decay instrument using a PRA spark lamp. All decays were best described by multiexponential decay. The paper appeared in Anal. Letters as a special review article. The dyes examined were anthracene isothiocyanate, three derivatives containing the dansyl moiety, two eosin labels, fluorescamine, fluorescein isothiocyanate, a maleimidylphenylcoumarin, two isomers of iodoacetamidofluorescein, various naphthalene-containing labels, lucifer yellow, maleimidyl salicylic acid, pyrenyl methyl iodoacetate, orthophthalaldehyde, quinacrine mustard, rhodamine B isothiocyanate, a stilbene isothiocyanate, and others. The lifetimes ranged from about 0.2 nsec for the stilbene derivative to over 56 nsec for the pyrene-containing label. These data should be a useful guide to the properties of these dyes attached to proteins.

Significance to Biomedical Research and the Program of the Institute:

Modern fluorescence methods utilizing lasers have extended the usefulness of emission spectroscopy. The instrument developed here is useful for studying the rapid motions constantly taking place on the molecular level. It is clear that many hitherto poorly understood phenomena can be elucidated by studying the rapid motions of flexible segments of molecules such as proteins. For instance, the fitting of antibody to antigen, the interaction of active sites with substrates and inhibitors, and the refolding of macromolecules all involve the rapid sampling of possible conformations - all on the picosecond to nanosecond scale. The present activity in instrumentational development and fluorescent reagent analysis is an extension of work supported for many years in this Institute on fluorescence spectroscopy, a technique which has led to many advances in biology and medicine.

Proposed Course:

The new Hewlett-Packard minicomputer will be interfaced with the laser-based nanosecond fluorometer. The full power of the instrument should then be available for rapid accumulation of time-resolved anisotropy and spectral data. Testing and application of the system will be done with Dr. Knutson. Additional spectral data on protein labels have already been obtained with Mrs. Scott, and similar data will be obtained on yet newer reagents and dyes which attach by adsorption rather than convalent attachment to proteins.

Publications:

1. Chen, R. F. and Scott, C. H.: Atlas of fluorescence spectra and lifetimes of dyes attached to protein, Anal. Lett. 18(A4), 393-421, 1985.

2. Knutson, J. R., Chen, R. F., Scott, C. S., and Bowman, R. L. "Studies of Intrinsic Protein Fluorescence Decay using a Modelocked Laser Source" Abstr. WAM-E4, Photochem. Photobiol. Vol 41, Supplement, p. 78S, 1985.

ff3

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01413-23 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Biophysical Methods for Study of Bio-Macromolecular Reactions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I. R. L. Berger Chief, Biophysical Instrum Section LTD:NHLBI

COOPERATING UNITS *(if any)*

Lab. Molecular Biology (J. Froehlich), Univ. of Pennsylvania (L. Thiebault), Biomedical Engineering & Instrumentation Branch (H. Casio, and Commonwealth Technology, Alexandria, Virginia.

LAB/BRANCH

Laboratory of Technical Development

SECTION

Biophysical Instrumentation Section

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	.5	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new inertial drive flow system has been developed for doing quench flow reaction kinetics in the investigation of the mechanism of reaction of Sacroplasmic Reticulum, ATPase, and other enzyme systems which cannot be followed by optical means. A time resolution of better than 1.5 ms has been achieved to date using the acid quenching of the reaction of 2-4 dinitrophenyl acetate as the test reaction. 0.25 ml of each reagent are required per data point in contrast to 2.5 ml for other instruments. Extension of its use for cryoquenching at -40°C is planned for the coming year. The use of this drive in our zero pressure drop thermal stopped flow system was reported last year. Pressure, velocity, and thermal measurements underway over a series of viscosities have provided information needed for the support of the ball mixer theory, i.e. that mixing is mainly the result of changing linear momentum to angular momentum. The results to date demonstrate that for a viscosity of one centipoise a pressure differential of less than 10 psi is sufficient to produce better than 99% mixing at 3 M/sec with thermal fluctuations of only ±2m°C. In addition optical fiber sensors will be added in the coming year so that simultaneous thermal-optical stopped flow experiments may be done.

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Project Description:

Objectives:

The major objective of the work of this section is to develop new instruments, analysis methods, and data handling techniques for the physiochemical study of biological reactions. The mechanism of a reaction, i.e. the underlying physical and chemical processes involved, is considered to be understood when a) the structural properties of the reacting molecules are known, b) the kinetic path and rate constants are known, and c) the thermodynamics are known In biological systems, a knowledge of how the structure-function relation works to regulate the rate of the reaction is also of vital interest as this is how catalysis is controlled. This section is particularly interested in model reactions because it is believed that considerable progress can be made in developing a more general theory of structure-function regulation if these reactions are understood in detail. The reaction of hemoglobin with various ligands and effector molecules (small molecules which alter the structural properties of the large protein molecule) is used as the principle model of a regulatory protein reaction. Other enzymes are used from time to time if they are available in the same high purity as hemoglobin. The reactions of various cellular enzymes, such as ATPase, both Na, K transport and Ca transport as well as calcium regulation in calmodulan, are studied as they relate to regulation.

Methods Employed:

Biological physics attempts to find the simplest explanation for complex systems. Toward this end model biological molecules are chosen which in general, are readily obtained highly purified and for which some structural information is available. Systems are chosen to work on for which several possible theoretical models exist to describe their mode of action. Methods are devised to provide the most accurate data possible to assist in deciding between these theories. Since the models chosen are of great biological interest, the instruments and methods generally become useful for the study of other systems as well. This section interacts with other laboratories in order to demonstrate feasibility of these models for their studies and assists commercial companies in producing these instruments for general use. The methods used in the investigation of the mechanisms of enzyme action are primarily those of pre-steady state chemical kinetics and thermodynamics. Measurements of the appropriate parameters are made by developing the necessary equipment to mix solutions rapidly and follow the course of resulting chemical reaction by optical, thermal, pH electrode, etc. detectors. In general, equipment to mix solutions rapidly and follow the course of the resulting chemical reaction by optical, thermal, pH electrode, etc. detectors. In general, equipment do to this is not available, either in the literature or commercially, for investigations in this area. Such apparatus is conceived and designed in this laboratory. Construction is carried out wherever most appropriate, i.e., in commercial firms, in other government agency shops, such as NRL or NBS, or at university shops. In pursuing these

investigations, a wide variety of physical parameters must be studied in order to understand the underlying physical theory governing the reactions. Expert consultants are used to assist where needed and biological collaborators join us to insure the proper handling of the biological molecules.

Major Findings:

The need to study reaction paths in macromolecular reactions becomes particularly important when the biological or chemical synthesis of a protein is contemplated. These details often can only be studied using so-called "pre-steady state kinetics". That is simply to say that if enzyme A reacts with substrate B to make C which reacts with a second substrate D to generate E details of A plus B, which is normally a very fast reaction and is thus treated as constant in the presence of large amounts of B and D, must be known. This fast step occurs in microseconds to milliseconds.

The development of a novel new flow apparatus called the "Inertial Drive Stopped or Quench Flow Apparatus" has been undertaken to overcome some of the problems of existing instruments. These problems include poor mixing of viscous solutions, stopping artifacts, and the use of large amounts of material in the case of the Quench Flow System.

A careful testing of the stopped and quench flow drive systems revealed several flaws in the hitchfeed design. Considerable effort has been expended in the last year to solve this problem. A completely new design has been developed which alleviates the problem of rapid acceleration of flow to a constant value and a fast clean stop. Combined flow velocity, pressure drop across the mixer, and temperature rise due to frictional heating and the adiabatic pressure heating of water have been measured in water. Measurement at different viscosities and flow velocities are presently underway.

Significance to Biomedical Research and the Program of the Institute:

Molecular biology and biological physics study the underlying physical and chemical processes involved in biological reactions at the cellular level. This section concentrates on developing new methods that will permit careful measurement of these reactions so that theories of the reaction mechanism can be critically tested. These theories will lead to a far better understanding of how the cell functions and how the fundamental catalysis which ultimately determine all bodily functions from synthesis to metabolism.

Proposed Course:

The time resolution of the quench flow apparatus will hopefully be pushed to its design value of 0.5 ms and a push-push operational mode developed. Optical fibers will be installed in the thermal stopped flow work started using instruments on various reactions.

ftb

Publications:

Balko, B., Bucci, E., Berger, R. L., Swarzendruber, L. J., and Montemarano, J. X.: Iron electronic structure in oxyhemoglobin and carboxypeptidase digested derivatives. J. Biochem. Biophys. Methods, 10, 55-64, 1984.

Smith, P.D., Liesegang, G. W., Berger, R. L., Czerlinski, G., and Podolsky, R. J.: A stopped-flow investigation of calcium ion binding by ethylene glycol bis (β-aminoethyl ether)-N,N'-tetracetic acid. Anal. Biochem. 143: 188-195, 1984.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01414-13 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Biocalorimeters for Solution and Cell Biochemical Studies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	R. L. Berger	Chief, Biophysical Instrum Section	LTD:NHLBI
	T. Kolobow	Medical Officer	LTD:NHLBI

COOPERATING UNITS *(if any)*

Biomedical Eng. & Instrumentation Branch, DRS, (C. Mudd), Northwestern Univ. (M. Marini), Penn State Univ. (N. Davids), N. M. Univ. School of Medicine (P. Simons), and Commonwealth Scientific, Alexandria, Va., Commonwealth Technology, Inc., Alexandria, Va.

LAB/BRANCH

Laboratory of Technical Development

SECTION

Biophysical Instrumentation Section

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.60	.60	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The black diamond coated polypropylene flow cell has been further improved to eliminate microscopic leaks that develop after extended use. Woods metal encapsulating has improved the thermal properties of the system. A small electrical heater installed on the surface of the reaction region permits calibration and heat capacity measurements. The installation of complete computer control, data collection, and analysis is presently underway.

Several biochemical reactions are presently being explored using the instrument. These include clatherin assembly, tRNA,-transferase reactions and ATCase reactions with various substrates.

The significance of the project lies in the possibility of detecting new reaction pathways as revealed by the thermal reactions and in the ability to make basic thermodynamic measurements on biological reactions where no other detection system is available. In addition, the direct measurement of the reaction enthalpy in a much more precise method than the determination by using the Van't Hoff method, i.e. measuring of the reaction equilibrium at different temperatures. The reason for this is that the range of temperature, 4°C to 40°C is to small for an accurate determination of the enthalpy. Thus to predict the change in reaction equilibrium, a very important quantity, in for example, drug metabolism, a direct calorimetric measurement is much to be preferred.

fff

Project Description

The objectives of this project are to develop methods of measuring the enthalpy of both solution and cell reactions. The many types of reactions which occur even in the simplest system must be identified so that the heat measured is, in fact, that produced by, say a binding reaction, and not the binding plus the heat of ionization of a titratable group which is titrated because a hydrogen ion is released by the reaction. This project is also aimed at utilizing the calorimeter as a modern Warburg respirometer with a thousand fold increase in sensitivity. This would allow detailed metabolic studies to be made including the effects of drugs on the reactions once the thermochemistry of these reactions is known. Thus, initial screening of pharmaceuticals could be done using liver, kidney, and other cells.

Methods Employed:

Utilizing various biochemical and cellular reactions, as a model system, appropriate instrumentation is designed and built in this laboratory where close interaction between investigator and instrument developer can be carried out. A close working relationship is maintained between this section and BEIB in the design and fabrication of new detectors, amplifiers, etc. At the same time wide use is made of commercial, academic, and other government agencies laboratories both to take advantage of the latest new technology and also to obtain, when possible, a significant savings for the government. This section specializes in being aware of the latest physical techniques being developed for other work and then adopting those methods, were appropriate, for biological studies. In addition to instrument development, considerable effort is put into data collection and analysis. Thus microprocessors are used when they will significantly improve the quality of the data taken. We have developed a simulation technique for analyzing very complex biological problems, particularly those involving heat conduction, or diffusion, and simultaneous chemical reactions. This is called the D-B Finite Element Simulation Technique or FEST. This method is employed in all of the systems developed.

Major Findings:

The study of the reactions of proteins with various ligands and effector molecules is necessarily dependent both upon advances made in protein purification and upon instruments to observe the reactions. Several important questions have gone essentially unanswered over the years more from a lack of instrumentation and of an understanding of the physical chemistry of proteins in solution than from the purity of the protein. One of these concerns the role that water plays in macro-molecular reactions. Water is gradually being recognized as a major constituent of the energy system which affects the interaction between all kinds of materials in solution. A good example is the interaction potential between DNA molecules as presently being elucidated by Parsegian, (DCRT). Basically, the question arises when a

protein or nucleic acid undergoes a confromational change upon reaction and the energy to drive this change does not appear to be available in the reaction bond energy. Two possible examples of this are the Ca^{-}calmodulin reaction with various proteins and the reaction of oxygen and hemoglobin. To address this problem we have been attempting to construct microcalorimeters which would on the one hand give the total heat of the reaction and on the other hand follow the thermokinetics of the reaction - Van't Hoff plots are not reliable since the determination of the equilibrium constant can be made over such a small temperature range with a protein. If structural changes occur the heat capacity of the system has changed and thus ΔH from Van't Hoff plots are meaningless. Why should there be an interest in the heats of reaction? The equation which governs all reactions in solutions and solids is that the Gibbs free energy, ΔG, that is, will the reaction "go" spontaneously and thus produce energy or does energy have to be supplied, is determined by knowing the equilibrium constant of the reaction.

$$\Delta G = -RTLnK$$

Under conditions of constant pressure ΔG is also equal to the change in the heat of reaction minus the change in entropy times the temperature, i.e.

$$\Delta G = \Delta H - T\Delta S$$

ΔS relates to the conformational changes that occur during the reaction. In the past this reaction scheme has expressly ignored water. The effects of salts may be included, but are generally also ignored. Thus if K and ΔH can be measured and the effects of water and salts sorted out the conformational change as given by ΔS can be determined. Eventually this can be correlated by x-ray, replacement or other methods of structure determination. Knowledge of these structural changes are particularly important when synthesis of a protein is attempted, or when drugs are being designed to interact with a protein to block enzyme activity such as in cholesterol synthesis, etc. This is also important in a multifunctional enzyme such as calmodulin when its interaction to control Ca activity in cardiac muscle may be entirely different form its control of Ca entry into nerve axions or brain cells.

The use of microcalorimetry in biology has been relatively small over the years due to the size and concentration of the sample needed, the sensitivity of the instrument, the many artifacts associated with thermal reactions, and the long time needed for sample equilibration. Often only two or three reactions can be performed per day. All chemical reactions either take up or give off some heat and thus microcalorimetry has the potential to be of great usefulness in studying biological processes, particularly in membrane and cellular reactions where often no optical change may occur.

In order to take advantage of this potential a new flow microcalorimeter has been developed in this section which promises to be of considerable use for biological reactions. Several reactions have been studied using the system

in the last few months. The most recent system has been the dissociation of NADASE dimer to monomer. The concentration of dimer in the 50 microliter samples used was 2.5×10^{-9} moles. The dissociation heat was about 2Kcal/mole. This gave 5 microcalories. At this point in the instrument development, we have a sensitivity of .1 microcalorie but small artifacts raise the variation to 1 or 2 microcalories. An hour is still needed for the initial equilibrium to be reached but reactions can be run every 15 minutes after that assuming they are complete in that time. tRNA-tRNA transferase reactions as well as ACTase reactions are planned in the coming months. Experiments with scientists in NIADKK on the assembly of protein molecules to form clathrin indicated a reasonable amount of heat per bond assuming the possible number of bonds formed if all the amino acids contributed. The overall heat per clathrin molecule formed i.e. 32 kelions, was 10^6 calories.

Significance to Biomedical Research and the Program of the Institute:

The major thrust of biomedical research is to understand the basic physical and chemical processes that go on in cells and tissues, link these processes to the overall physiological functions of the living organisms and thus better enable the practicing physician to keep the population healthy and more intelligently treat the patient when there is illness. The instruments being developed under this project will permit the biomedical investigator to determine the thermodynamic constants that are needed to develop a correct theory for the uptake and exchange of oxygen, carbon monoxide, carbon dioxide, etc. in the blood, tissues, and lungs. Calcium is an important regulator of cellular function and the constants which will be determined with these instruments will ultimately lead to a better understanding of how this regulation takes place. The manner in which enzymes work with DNA to do what the cells need to do for synthesis is one of the fundamental problems of molecular biology and just as in organic chemistry, the details of these reactions must be known if they are ultimately to be used in the vast number of genetic applications now being started in this rapidly growing new field.

Proposed Course:

The batch calorimeter, .3 ml cell, will be instrumented for use with cells. Optical pH, pCO_2, and pO_2 probes will be introduced along with lines for various titrants to add substrate and inhibitors. We well thus be able to run the system as a Warbug respirometer but with 10^3 to 10^4 fold increased sensitivity. The flow calorimeter will be further improved to eliminate all artifacts and noise and by using an all tantalum mixer flow system bring the sensitivity level to a point where a 5 µcal reaction can be measured to $\pm$ 2%. This would make it possible to determine the enthalpy of biological reactions at realistic concentrations of enzyme, i.e. 10^{-6} M.

Publications:

Berger, R. L., Cascio, H. E., Davids, N., Gibson, C. G., Marini, M., and Thiebault, L.: An automated differential thermal and potentiometric titration apparatus for binding studies. J. Biochem. Biophys. Methods, 10, 245-259, 1985.

Davids, N., Berger, R. L., and Marini, M.: Application of the finite element simulation method to the adiabatic and potentiometric corrections of calorimetric titration data. J. Biochem. Biophys. Methods, 10, 261-272, 1985.

Marini, M. A., Evans, W. J., and Berger, R. L.: Use of the twin-cell differential titration calorimeter for binding studies. I. EDTA and its calcium complex. J. Biochem. and Biophys. Methods, 10, 273-285, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01421-10 LTD

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development of Electrochemical and Physiological Methods for Cell Research

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.:	R. E. Steele	Physical Science Investigator	LTD:NHLBI
Others:	R. L. Bowman	Chief, LTD	LTD:NHLBI
	M.R. Kindt	Chemist	LTD:NHLBI
	J. W. Handler	Section Chief	KE:NHLBI

COOPERATING UNITS *(if any)*

Laboratory of Kidney and Electrolyte Metabolism, NHLBI

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The porous bottom culture dishes (PBCDs) and related devices developed in this laboratory for the sterile measurement of electrophysiological parameters of cell layers are used in over 50 laboratories in the U.S.A. Two companies have devoted considerable effort toward producing commercial versions of the PBCDs using cellulose ester membranes (Millipore Corp.) and plasma treated polycarbonate membranes (Becton-Dickinson Corp.). Many types of epithelial cell layers are being grown on the PBCDs using a variety of membrane materials. Recently, endothelial cell layers have been grown here at NIH on our PBCDs with transparent collagen membranes. A defined medium has been developed for use with the PBCDs to optimize a differentiated function (transepithelial Na transport) rather than to maximize growth. Since the PBCDs facilitate the study of this differentiated function, this medium has obvious advantages.

The major role played by Ca in the regulation of many cellular processes has prompted us to improve methods of measuring free Ca activity inside cells. microelectrodes need to be as small as 0.1 microns in diameter to enter many cells without causing damage which makes reliable readings extremely difficult. Electrodes of this size have walls so thin that they may be totally hydrated near the tip and become Na and K electrodes due to the monovalent metals contained in the glass. This conflict between Ca, Na, and K sensitivities is believed to cause the poor performance often seen for electrodes smaller than 1 micron in diameter at Ca activities below micromolal. We have therefore designed, constructed, and used unique anular burners for making micropipettes to fused quartz which is free of metals other than the silicon of the silicone dioxide. An improved silanization method is being developed for these pipettes so that the advantages of the quartz can be fully realized.

Objectives:

(1) Develop instruments and methods to make possible the study of cells in culture in ways that have not been done before.

(2) Improve methods for the measurement of intracellular Ca^{++} activity of individual cells. Measurements in epithelial and endothelial cells in culture are our prime objectives.

Methods and Results:

(1) Endothelial cells have been grown on collagen membrane porous bottom culture dishes (PBCDs) made in our laboratory. The cells have been put on the PBCDs by Dr. Dan Malone (two types of small vessel endothelial cells) and by Dr. Dan Rotrosen (large vessel endothelial cells) both of the NIAID. The cells have all grown to confluence and we have been able to measure resistance using our devices for sterile electrical measurement. Preliminary results indicate that these preparations have a very low electrical resistance under the conditions used. We are greatly encouraged by these early results and anticipate learning many new things about endothelial cell layers.

(2) Two companies in the country have devoted effort toward producing commercial versions of our PBCDs. Millipore Corp. has made two prototype batches and one production scale run of PBCDs using their cellulose ester membranes. None of these have been entirely right, but they are working actively on solving the problems. In the past, Becton Dickinson Corp. developed PBCDs that performed very well using plasma treated polycarbonate membranes. The increased interest in cell culture using PBCDs may cause them to resume work on these devices.

(3) The PBCDs and related devices facilitate the study of a particular differentiated cellular function by epithelial cells: transepithelial sodium transport. We have developed a defined medium with the objective of optimizing this differentiated function by A6 cells from Xenopus laevis kidney. Unlike the majority of defined media, ours contains no insulin and it is not our objective to achieve the maximum possible growth rate. The composition of the FBS replacing material in final concentrationis:

Epidermal growth factor	$1.5 \cdot 10^{-9}$	molar
Transferrin	$5.5 \cdot 10^{-8}$	molar
Hydrocortisone	$5 \cdot 10^{-8}$	molar
Triiodothyronine	$5 \cdot 10^{-12}$	molar
Prostaglandin E_1	$5 \cdot 10^{-8}$	molar

The activity of calcium can be better controlled in this medium than in a serum supplemented medium. We expect this feature will be useful for our planned calcium studies. For the A6 cells, the growth surface must be treated with fibronectin (12 $\mu g/cm^2$ for cell attachment). The composition of most defined medium are adjusted to achieve maximum growth rates. We adjusted ours to achieve stable and long lasting transepithelial sodium transport. Maximum sodium transport was not even desired since that might preclude subsequent stimulation by hormones such as vasopressin, aldosterone and insulin. The criteria of sodium transport stability and hormonal responses take longer to evaluate that just cell growth. At the present time, we have a satisfactory medium for the study of transepithelial sodium transport by A6 cells and it serves as an example media for the study of differentiated function by other cells.

(4) We have been making micropipettes for micro Ca^{++} electrodes from fused quartz tubing using our unique anular burners installed in two commercial micropipette pullers. The silanization of tips smaller than 0.3 micron in diameter with monomolecular layers of chemically attached silanes is difficult. Tips of this size cannot be resolved with a light microscope and the capillary forces in such tips are large. This washing away of excess silane with an organic solvent free of water is a considerable but worthwhile challenge. An excellent job of silanization is probably required to achieve the advantages of the fused quartz micropipettes.

Significance to Biomedical Research and the Program of the Institute:

The apparatus and method necessary to grow and study sheets of epithelial cells on membranes is advancing the study of the basic mechanisms of active Na transport. The ability to make the measurements under sterile conditions greatly increases the productivity of this work. The growth of the cells on nutrient and gas permeable membranes results in a degree of development and differentiation which does not occur on the conventional plastic or glass surface. This should allow a study of the development of epithelial cells and endothelial cells that is not possible otherwise. The accurate measurement of the Ca^{++} activity inside cells at various stages of development should greatly increase our knowledge of cell physiology and differentiation.

Proposed Course:

(1) Design a sterile cell growth system for epithelial or endothelial cells such that cells form layers on the inside of collagen tubes which are continuous bathed on the inside and the outside by flowing medium. The following advantages are expected:

(a) The medium composition should be constant, rather than varying widely as it does when feeding is done 2 or 3 times per week.

(b) The gas composition can be controlled closely even at very low oxygen concentration for ischemia studies.

(c) The flow rate of the medium over the cells will be uniform.

(d) The cells may be viewed with a phase microscope through the walls of the collagen tubes.

(2) Develop electrodes to measure Ca^{++} activity in epithelial and endothelial cells which have tips smaller than 0.3 microns and low interferences so that Ca^{++} activities of 10^{-6}, 10^{-7}, 10^{-8} and even 10^{-9} molal can be measured correctly.

(a) Silanize pipettes in flow through system with long chain monochlorosilanes.

(b) Coat outer surface of microelectrode in such a way as to facilitate entry into cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01435-06 LTD

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: New Catheter Idea to Facilitate Radiologic Instrumentation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	R. L. Bowman	Chief, Lab. Technical Development	LTD:NHLBI
Others:	R. Paul	Physical Science Technician	LTD:NHLBI
	J. Doppman	Chief, Radiology Department	DR:CC

COOPERATING UNITS *(if any)*

Diagnostic Radiology Department, Clinical Center, NIH

LAB/BRANCH: Laboratory of Technical Development

SECTION:

INSTITUTE AND LOCATION: NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	1	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The concept of a topocatheter extension added to a standard angiographic catheter involves the production of a high strength thin walled tapered tube attached to the end of a standard catheter. Suction on the catheter causes the thin wall portion to collapse and invert itself until it lines the distal end of the catheter. Flow out of the catheter causes the thin wall to valve shut and evert again. When the thin wall everts it rolls onto the inside of the vessel wall with no relative wall motion hence no friction. The rolling wall is advanced by the pressure of the fluid inside so that the extension enters and follows small or tortuous vessels with ease.

We are continuing to supply collagen extension catheters to Dr. Doppman but other applications that do not require protection from ballooning and obstruction of flow can be met by returning to the plain polyurethane thin wall catheter.

New tubing of high quality Tecoflex has been drawn to produce higher quality catheters and solution casting or dip coating have been demonstrated to produce catheters that promise easy and rapid fabrication so that made to order devices can be tailored to specific clinical problems.

In addition to catheter production from collagen the tubes made have been slightly modified to make 3mm tubes that have been supplied to the surgical service for testing as vascular prosthesis. The strength texture and suture holding qualities have been reported to be superior and animal implants have remained patent for a short time which is relatively encouraging for this size vessel.

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Project Description:

Objectives:

To improve the strength and performance of the extension everting angiographic catheters and characterize the performance specifications required for safe human therapeutic applications.

Methods Employed:

We have continued to test other formulations of collagen and other polyurethanes using the established method which uses multiple coats of collagen applied to rotating lass forms and finally dip coating in solutions of polyurethanes.

The extension portion of the catheter is prepared by coating a dilute collagen dispersion (Ethicon TD 192) on to a rotating tapered glass mandrel. The dilute dispersion is prepared by blending 1 part 50:50 water methanol with 1 part Ethicon collagen dispersion and adding sufficient acetic acid to keep the collagen in solution. Several coats are applied and allowed to dry in air. The collagen coated mandrels are then soaked in .1 M ammonium hydroxide for 8 hours. They are then thoroughly rinsed in distilled water and transferred to .1% formaldehyde for several minutes then rinsed repeatedly in distilled water. They are dried at 55°C in dry CO_2. The dry forms are then dipcoated in a 4% solution of medical grade Tecoflex (Thermo Electron Corp.) in N,N,Dimethylacetamide (Eastman Kodak Chemical Co.) and maintained at °50C. They are then cycled through several rinses with distilled water.

After removal from the mandrel under distilled water they are attached to standard polyethylene agiocatheters (Becton Dickinson Formocath polyethylene tubing) by slipping the collagen-urethane sleeve over a previously etched end of the PE catheter. The PE catheter is wedged into the tapered catheter and secured in place by a polyurethane sleeve which is secured tightly over one extension and the PE catheter. The joint is cemented with the same polyurethane solution as was used to coat the collagen. The dry catheter with extension is Et_0 gas sterilized in the usual way.

Two alternate methods of producing the extension that were used previously and abandoned because they distended under pressure to fill the entire lumen of the artery. In the brain this was unacceptable but for treatment of vascular anomalies it is not only acceptable but much easier to make.

One method simply dipcoats tapered glass forms that were flame worked to provide the size and shape required. The coating of Tecoflex polyurethane is easily removed from the form and attached to the angiocatheter. Another

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simple rapid method involves drawing Tecoflex polyurethane tubing in a machine constructed for this purpose.

The small diameter vascular grafts are prepared as follows:

Stock collagen dispersion supplied by Ethicon Corp., Sommerville, N.J. is diluted with an equal amount of water-methanol 50:50. The resultant mixture is collagen dispersion:water:MeOH (50:25:25). This mixture is then blended in a high speed blender Virtis "45" at medium speed for 3 min. approximately 20,000 rpm. Air bubbles are allowed to settle out before use. The collagen solution is then brushed on with a 7mm camel hair brush, using longitudinal strokes applied to cylindrical glass mandrel rotated at 120 rpm. When each coat dries (20 min) another coat is applied.

After sixty coats are applied the collagen is precipitated in 3.5% ammonium hydroxide or aqueous solution of 4% chondroiton-6-sulfate. The precipitated collagen is then crosslinked in .05% foraldehyde or .01% glutaraldehyde for 1/2 hour.

The precipitated, crosslinked collagen coated mandrels are then allowed to dry in flowing CO_2 at 40°C for several hours. They are then dip coated in 5% Tecoflex polyurethan (85A) in N,N-dimethylacetamide (Fisher, certified) dried and dipped again in the same solution this time being immersed in warm 0.9% salt solution immediately after the polyurethane dip. This treatment causes the surface of the polurethan to foam and turn opaque white, it is dried and another coat is applied without the salt treatment, the mandrel is then dried in circulating air at 50°C. The mandrels soak in saline overnight, are then removed the next morning by sliding the mandrel out of the tube.

The resultant tube is then inspected for size and quality of the inner surface (collagen side). Radial and longitudinal compliance to change an internal pressure is then measured. Good sections are chosen and sterilized by ethylene oxide.

Major Findings:

Successful construction and application of the extension catheter has been demonstrated in the Department of Radiology where continued work will be needed to establish its place in clinical diagnosis and therapy. Theeversatility of the methods permit satisfactory catheter to be made that have burst pressure greatly exceeding eversion pressures. In the vascular modification proline sutures break before they pull though the walls. Implanted vessels remain patent long enough to indicate a lack of thrombogenic effect but ultimately block at the 3mm size.

Significance to Biomedical Research and the Program of the Institute:

Intravascular diagnostic and therapeutic catheters are essential to heart and vascular research. The need for a source of small vascular prosthesis has not been adequately met.

Proposed Course:

The production of catheters is at the stage where it should be taken over by a facility that can standardize production and sterilization with adequate controls and inspection to relieve this laboratory of production problems so that we can return to research on application to other problems including vessel prosthesis. Several catheter companies have expressed some interest but one has constructed some of the production tools and is ready to supply catheters made by the presently described methods (Cook Co., Inc., Indianapolis, IN).

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01445-04 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Large Preparative Countercurrent Chromatography for Separation of Polar Compounds

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	Y. Ito	Senior Investigator	LTD:NHLBI
	J. Sandlin	Biologist	LTD:NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.3	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Gram quantity separation of polar compounds (trp-leu and val-tyr) was achieved with a hydrophilic solvent system composed of n-butanol acetic acid/water (4:1:5) by the use of a large diameter coiled column (0.55 cm i.d.) on a horizontal flow-through coil planet centrifuge described in the previous report (Z01 HL 01445-03 LTD). Using a short coiled column, stationary phase retention and peak resolution were studied under various operational conditions by altering helical diameter, rpm, flow rates, and elution mode of the mobile phase. Under the optimum operational conditions (300 rpm and 120 ml/h flow rate) preparative-scale separations were performed on 1g quantity of samples with a long column (30 m in length, 750 ml capacity and 7.5 cm helical diameter). The sample-loading capacity of the present system may be further increased by the use of longer and/or larger-diameter columns.

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Project Description:

Objectives:

Preparative-scale separation of polar compounds with a hydrophilic solvent system by large preparative high-speed countercurrent chromatography.

Methods Employed and Major Findings:

The present series of studies were performed with a bench top model of the horizontal flow-through coil planet centrifuge described in the previous report (ZO1 HL 01445-03 LTD).

1. Preliminary Studies on the Short Column:

A series of preliminary experiments were conducted with short coiled columns of 7.5 cm and 15 cm helical diameters, each prepared from a 420 cm long, 0.55 cm i.d. FEP tube with a total capacity of 114 ml. The partition performance of these columns were examined on a hydrophilic solvent system composed of n-butanol/acetic acid/water (4:1:5) for separation of two dipeptide samples, L-tryptophyl-L-leucine (trp-leu) and L-valyl-L-tyrosine (val-tyr). In each separation, the column was first entirely filled with the stationary phase followed by sample injection through the sample port. Then the apparatus was rotated at a given revolutional speed while the mobile phase was pumped into the column. Effluent from the outlet of the column was continuously monitored with a Uvicord S at 280 nm and then fractionated into test tubes. After the two peaks were eluted out, the apparatus was stopped and the column contents were collected into a graduated cylinder to measure the volume of the stationary phase retained in the column. The experiments were repeated by varying rpm (50, 100, 200, 300, and 400), flow rates (120 ml/h and 240 ml/h), and the elution modes (head to tail and tail to head) using both phases as the mobile phase.

The results are summarized as follows:

(1) The present system always distributes the lower aqueous phase toward the head and the upper nonaqueous phase toward the tail of the coil.

(2) The best retention and peak resolution were obtained by either the head to tail elution of the upper phase or the tail to head elution of the lower phase.

(3) The highest peak resolution we produced from the smaller helical diameter (beta = 0.25) at a revolutional rate of 300 rpm and the slower flow rate of 120 ml/h.

2. Preparative-scale separation with the long column:

Preparative capability of the present system was evaluated on separation of various quantities (100 mg, 250 mg, 500 mg and 1 g) of the sample mixture

with a long coiled column consisting of a 30 m long, 0.55 cm i.d. FEP tube with a total capacity of 750 ml. Separations were carried out under the optimum operational condition determined by the above preliminary studies. The results showed that both stationary phase retention and peak resolution were gradually decreased with the increased sample size but, even at the maximum dose of 1 g, the peaks are well resolved. Overall results indicated that the present method is capable of efficiently utilizing the hydrophilic butanol solvent system which is extremely useful for the separation of various polar compounds. The sample-loading capability of the system may be further increased by the use of longer and/or larger-diameter columns.

Significance to Biochemical Research and the Program of the Institute:

Separation of polar compounds often produces various problems in HPLC and other separation methods. Successful purification of a large quantity of such compounds with high purity and good recovery is extremely important in many biochemical researches.

Proposed Course:

1. Addition of a temperature control system to the apparatus to improve the partition efficiency of the column.

2. Application of more hydrophilic solvent systems such as sec.-butanol/water for separation of extremely polar compounds.

Publications:

Sandlin, J. L. and Ito, Y.: Large Preparative-Scale Countercurrent Chromatography with a Coil Planet Centrifuge, J. Liq. Chromatogr., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01449-03 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

Monolithic Integrated Countercurrent Chromatography (MICCC)

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigetor.) (Neme, title, laboretory, and institute affiliation)*

P. I.	T. Kolobow	Medical Officer	LTD:NHLBI
	Y. Ito	Medical Officer	LTD:NHLBI
Others:	J. Morabito	Biol. Lab. Techn.	LTD:NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

Section on Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	.25	.75

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

We have designed a monolithic integrated countercurrent chromatography (MICCC) system for the continuous or batch separation of solutes, particles, or cells. We are using differential solubility of compounds in a two phase solvent system as the principle in chemical compound separation. The MICCC system represents a multistage system embodying this principle, and is formed of a suitable plastic, or metal. Separation is enhanced when MICCC is vibrated, shaken, or otherwise agitated for enhanced mixing.

The centrifugal MICCC was devised to greatly increase the capacity of MICCC by providing improved mixing, and excellent phase retention. Such a device can be particularly useful for particle, and cell separation.

Objectives

It is the purpose of this project to devise an integrated system for the separation of solutes or biologic particles, either continuously or in a batch form. Such accomplishment could result in the cleaner separation of biologic compounds and particles of interest, and may lay the foundation to future advances in biology and medicine.

Methods Employed

We have devised an integrated flow system as part of a centrifuge rotor, that borrows some of the concepts from a commerically available cell elutriator.

In its present form, the centrifugal monolithic cell elutriator is formed of two shaped sheets of polypropylene, fused together. There are 40 individual conical locular cells of 4 ml each. The locules are interconnected in molded channels. The interconnecting tubes are arranged so that the feeding tube delivers feedstock to the most distant site from center of rotation, hence limiting or eliminating sedimentation of particles. The rotor is designed to be disposable after a single use.

Major findings

As in the commercial cell elutriator system, the Coriolis forces generated by the rotating centrifugal forces are also to be contented with. Stabilization of flow, and hence overcoming the Coriolis forces, was accomplished through the use of a gradient of either glucose or albumin. Such stabilization was essential to utilize the greatly enhanced capabilities of the multilocular rotor.

In pilot studies using mixtures of sheep and human red blood cells (5 and 7 micrometers, respectively), separation into two population groups was reasonably well accomplished. The separation of cell types of current interest to the research community is now being explored.

Significance to biomedical research and the program of the institute

Current scientific activity in cellular biology and cellular biochemistry requires the separation of subpopulations of cells from tissue or tissue cultures. The centrifugal multilocular monolithic integrated flow system is likely to emerge as an important contender to fill such a need.

Proposed Course

We shall further explore the potential of currently used centrifugal multilocular monolithic integrated flow sytem in the separation of cells and cellular components of interest to the local research community. Such collaboration will lead to enhanced understanding of the potential and the limitations of the current design, and point the direction for future improvements.

Publications

1. Kolobow T., Ito Y., Mychkovsky I., Peters P., Morabito J.: Monolithic integrated flow circuit (MIFC): a new column designed for countercurrent chromatography. Journal of Liquid Chromatography, 1985, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01450-02

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 cherecters or less. Title must fit on one line between the borders.)*

High-Speed Countercurrent Chromatography Under Elevated Temperature

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	Y. Ito	Senior Investigator	LTD:NHLBI
	M. Knight	Research Associate	ETB:NINCDS

COOPERATING UNITS *(if any)*

Experimental Therapeutic Branch, NINCDS (Dr. Martha Knight)

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	0.7	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

As proposed in the previous report (Z01 HL 01450-01 LTD), a temperature control system was added to our prototype coil planet centrifuge to improve partition efficiency of butanol solvent systems in high-speed countercurrent chromatography. A set of electric heating pads was pasted onto the inner wall of the centrifuge while the whole unit was insulated with foam plastic sheet. The column temperature became continuously adjustable from room temperature to 60°C with a high stability. The capability of the apparatus was demonstrated on purification of two types of crude synthetic peptides with suitable butanol solvent systems under elevated temperature of 50°C. The obtained fractions were analyzed by reversed phase HPLC to determine the purity. The results indicated that the present system yields high purity fractions (90%-98%) in short periods ranging from 30 minutes to 2 hours.

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Project Description

Objectives:

Development of a temperature-controlled coil planet centrifuge for high-speed countercurrent chromatography (CCC) and its application for purification of polar compounds with hydrophilic solvent systems.

Methods Employed and Major Findings:

Apparatus:

The present study employed a compact table top model of a coil planet centrifuge for high-speed CCC used in the previous experiments (ZO1 HL 01455-02). A temperature-control system was added to the apparatus as proposed in the previous report. A set of electric heating pads was pasted onto the inner wall surface of the centrifuge while the whole unit was covered with foam plastic sheet. With a temperature control unit (RFL Industries, Inc., Boonton, NJ) the temperature became adjustable from 25°C to 60°C with high stability. The column consisted of a 130 m long, 1.6 mm i.d. PTFE tube of multilayer configuration with beta values ranging from 0.5 at the internal terminal to 0.8 at the external terminal. The total capacity of the column measured approximately 280 ml.

Purification of Peptides:

The performance of the apparatus was examined on purification of crude synthetic peptides with butanol solvent systems. A cholecystokinin fragment (Ac-Asp-Tyr-Met-Gly-Trp-Met-Asp-NH_2) with n-butanol/0.2M ammonium acetate (1:1) and bombesin with n-butanol/dichloroacetic acid/water (100:1:100). Before the experiment the two-phase solvent system was equilibrated at 50°C in a container immersed in a water bath. In each purification the column was first entirely filled with the stationary phase and the sample solution was injected through the sample port. Then the apparatus was run at 800 rpm while the mobile phase was eluted through the column at a flow rate of 150 ml/h. During the run the temperature of the centrifuge was set at 50°C while the reservoir of the mobile phase was kept in a water bath at 50°C to maintain the phase equilibrium. The effluent from the outlet of the column was continuously monitored with a Uvicord S at 280 nm and fractionated with a fraction collector. The collected fractions containing the aimed peptides were analyzed with reversed phase HPLC to evaluate the purity.

Results:

In purification of the cholecystokinin fragment peptide (15 mg), the main peak containing the desired peptide was eluted early in 30 minutes near the solvent front, but it was fairly well resolved from the neighboring peaks. HPLC analysis of the fractions showed 90% purity. In purification of bombesin (120 mg), the partition coefficient value of the peptide was

adjusted close to 1 so that the main peak is retained much longer within the column to produce high peak resolution. Under these circumstances elution of the bombesin peak took place in 2 hours but a much higher purity of 98% was attained. The purification of the crude bombesin was also successfully performed with a solvent system composed of n-butanol/trifluoroacetic acid/water (200:1:200) which provides an advantage over the above solvent system in that the fractions can be directly lyophilized without pre-extraction of the acid with ether.

Significance to Biomedical Research and the Program of the Institute:

Many biologically active peptides and other compounds possess a high polarity and require the use of hydrophilic solvent systems for separation and purification with countercurrent chromatography. Performing countercurrent chromatography at elevated temperature renders the following advantages:

1. Application of a higher flow rate.

2. Higher partition efficiency due to reduced viscosity.

3. Increased sample-loading capacity due to increased solubility.

Therefore, the present system will be extremely useful in separation and purification of various biological samples.

Proposed Course:

Hydrodynamic studies of the motion of the two-phase solvent systems in a angle-rotor coil planet centrifuge at room temperature and elevated temperatures.

Publications:

1. Ito, Y.: Experimental Observation of the Hydrodynamic Behavior of Solvent Systems in High-Speed Counter-Current Chromatography. I. Hydrodynamic Distribution of Two Solvent Phases in a Helical Column Subjected to Two Types of Synchronous Planetary Motion, J. Chromatogr. 301: 377-386, 1984.

2. Ito, Y.: Experimental Observations of the Hydrodynamic Behavior of Solvent Systems in High-Speed Counter-Current Chromatography. II. Phase Distribution Diagrams for Helical and Spiral Columns, J. Chromatgr. 301: 387-403, 1984.

3. Ito, Y. and Conway, W. D.: Experimental Observations of the Hydrodynamic Behavior of Solvent Systems in High-Speed Counter-Current Chromatography. III. Effects of Physical Properties of the Solvent Systems and Operating Temperature on the Distribution of Two-Phase Solvent Systems, J. Chromatogr. 301: 405-414, 1984.

4. Knight, M., Ito, Y., Kask, A. M., Tamminga, C. A., and Chase, T. N.: Chromatography of Ac-Asp-Tyr-Met-Gly-Trp-Met-Asp-NH_2 on the Horizontal Flow-Through Coil Planet Centrifuge and the High-Speed Multi-Layer Coil Planet Centrifuge. J. Liq. Chromatogr. 7: 2525-2533, 1984.

5. Knight, M., Ito, Y., Peters, P., and diBello, C.: Rapid Purification of Synthetic Bombesin by Countercurrent Chromatography on the Multi-Layer Coil Planet Centrifuge, J. Liq. Chromatogr., in Press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01451-02 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Electron Spin Resonance Development for Medical and Biological Problems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	J. Zweier	Staff Fellow	LTD:NHLBI
Others:	R.L. Bowman	Chief, LTD	LTD:NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of this project is to develop and adapt electron spin resonance spectroscopy to study the biochemistry, physiology, and pathology of cells and tissues in order to answer problems of medical and biological importance. To accomplish this goal we are working on approaches to increase the sensitivity and developing cavity design suitable for different problems ranging from microsamples, to cultured cells to whole tissues. By utilizing different frequency microwave sources it is possible to optimize resonator design for each type of biological sample. Initially we assembled an X-band, 9 GHz spectrometer. Various resonators were designed and tested at X-band including several loop gap resonators. In order to accommodate large aqueous samples such as living perfused organs work was started on development of L band and S band spectrometers. An S band loop gap resonator was designed and built to enable the study of free radical generation in living perfused hearts.

Over the past year we have focused on 2 important cardiovascular applications (1) the mechanism of the adriamycin cardiomyopathy (2) the mechanism of ischemic and reperfusion heart damage. We demonstrated that Fe(III) binds to adriamycin and that these complexes cycle to reduce oxygen. Adriamycin reduces its bound Fe(III) to Fe(II) which then donates an electron to molecular oxygen. This mechanism explains the formation of reduced oxygen and drug radicals which are thought to mediate adriamycin's therapeutic and toxic effects. Free radicals are thought to be generated in the ischemic and reperfused heart and to mediate the cellular damage which occurs. We developed a direct ESR technique to measure free radical generation in the ischemic and post ischemic heart. These studies demonstrate marked free radical generation on reperfusion of the post ischemic heart.

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Project Description

Objectives:

The overall goal of this project is to develop and adapt Electron Spin Resonance Spectroscopy to study the biochemistry, physiology, and pathology of cells and tissues in order to answer problems of medical and biological importance. Electron Spin Resonance Spectroscopy is a powerful technique for studying metal metabolism and free radical generation. For paramagnetic metal ions such as Fe(III), Cu(II), Mn(II), and Co(II) information can be obtained about the redox state of the metal, the spin state, the symmetry of metal binding and the identity of binding ligands. For free radicals information can be obtained about their structure, mobility, stability, and quantitation. The Electron Spin Resonance technique provides critical information on these problems which cannot be obtained in any other way. It has been applied to a large number of biological problems at the in vitro biochemical level, however, the great potential of this technique has not been fully realized due to limitations in the sensitivity and sample geometry of conventional commercial spectrometers. With conventional commercial spectrometers only samples in 1-3 mm tubes or flat cells can be studied and the sensitivity is limited to 10^{-4} - 10^{-5} M concentrations of metal ions such as Fe(III) or Cu(II) or 10^{-6} M concentrations of free radicals. In order to study in vivo metal metabolism and free radical generation in intact cells and tissues it is first necessary to be able to accommodate the geometries of these biological samples and then the sensitivity must be optimized in order to be able to detect the concentrations of metals and radicals found within cells. Therefore, we are working on approaches to increase the sensitivity and developing spectrometer and cavity designs suitable for a large range of biological problems ranging from microsamples, to cultured cells, to whole tissues. While developing these techniques we are demonstrating their feasibility and importance by actively applying them to solve important medical and biological problems.

Methods Employed:

Over the past 16 months an X-band, 9 GHz, Electron Spin Resonance Spectrometer has been assembled. Various resonator designs were evaluated using this X-band spectrometer system including rectangular TE cavities, cylindrical TE and TM cavities, helical resonators, and loop gap resonators. Work was started on the development of L band, 1-2 GHz, and S band, 2-4 GHz, spectrometer systems designed to study large aqueous samples such as living perfused organs. We designed and built a large-gap resonator 26mm in diameter capable of accommodating a perfused rabbit heart. This resonator had a resonant frequency of 2.7 GHz with a Q of approximately 500 and it was free of any spurious resonances. The Q of this resonator is not significantly lowered when the resonator is filled with a 25 mm tube containing water or physiologic saline. This performance is particularly impressive when compared to that of the conventional TE102 cavity, at X-band,

whose Q decreases over 100 fold on changing from a 1 mm tube to a tube only 3 mm in diameter. In addition with the loop-gap resonator we can achieve a filling factor of 92% which is far better than the filling factor of less than 5% for the TE102 cavity. With this resonator it should be possible to study free radical generation non-invasively, non destructively in whole living perfused hearts. This is of great importance since free radicals are thought to cause myocardial damage which occurs during ischemic and post-ischemic reperfusion. This ESR technique would provide the only direct non invasive method to measure free radical generation in the heart.

Major Findings:

Over the past year we have focused on 2 important cardiovascular applications (1) the mechanism of the adriamycin cardiomyopathy (2) the mechanism of ischemic and post ischemic heart damage.

Adriamycin is one of the most potent anticancer drugs in clinical use but it is also very toxic to the heart and induces a cardiomyopathy which is the third most common cardiomyopathy in the United States. Numerous studies have suggested that the therapeutic and toxic effects of adriamycin are due to the formation of reactive oxygen and drug free radicals but the mechanism of this radical formation was unknown. Using the ESR we demonstrated that Fe(III) binds to adriamycin and that these complexes cycle to reduce oxygen. Adriamycin reduces its bound Fe(III) to Fe(II) with the formation of an oxidized adriamycin free radical. The Fe(II) then donates an electron to molecular oxygen forming superoxide and hydrogen peroxide. This mechanism explains the formation of reduced oxygen and drug radicals which are thought to mediate adriamycin's therapeutic and toxic effects.

Free radicals are thought to be generated in the ischemic and reperfused heart and to mediate the cellular damage which occurs. We developed an ESR technique to directly measure free radical generation in the ischemic and post-ischemeic heart. These studies demonstrate marked free radical generation in the heart during post ischemic reperfusion and provide the first direct evidence for free radical generation in the post ischemic heart.

Significance to Biomedical Research and the Program of the Institute:

Our Electron Spin Resonance development program is specifically oriented toward developing instrumentation and techniques to solve important biomedical problems. Electron Spin Resonance can yield critical information which cannot be obtained with any other available techniques.

Important groups of applications range from the study of respiratory electron transport in normal and damaged cells, as in myocardial ischemia, to studying the cause of carcinogenesis, to determining the mechanism of action of anticancer drugs, to the study of hematopoesis, to the study of drug

metabolism and the P450 enzyme system, to the study of transition metal metabolism and toxicity, to the study of cellular receptors proteins and membrane fluidity, to even the study of alterations in cells due to aging.

Over the past year this program has led to the solution of two important biomedical problems. A mechanism has been determined which can explain the origin of the adriamycin induced cardiomyopathy, which is the third most common cardiomyopathy in the United States (Zweier, J. Biol. Chem. 259, 6056-6059). This work may also provide important information regarding the tumoricidal mechanism of adriamycin.

We developed a technique to directly assay for the formation of free radicals in the heart. This technique is based on ESR measurements performed on samples from freeze clamped hearts. Using this technique we demonstrated that there is marked free radical generation on reperfusion of the post-ischemic heart. It has been proposed that free radical formation is the key mechanism of ischemic and post ischemic heart damage. Our studies provide the first direct demonstration and quantitation of free radical generation in the post-ischemic heart.

Using this ESR technique it should be possible to chemically characterize which radicals are generated, the enzymatic mechanism responsible for their generation, and to determine what pharmacologic interventions would be most successful at preventing post-ischemic reperfusion heart damage. This work is of great significance since ischemic heart disease and myocardial infarction is the number one cause of morbidity and mortality in the United States.

Proposed Course:

Over the next year we plan to continue our work on the development of L and S band spectrometer systems. We will continue to perfect resonator designs to obtain optimal sensitivity on biological samples. Work will be focused on developing a noninvasive, nondestructive ESR technique to assay for free radicals in the perfused heart.

Publications:

1. Zweier, J. L.: Reduction of O_2 by iron-adriamycin. J. Biol. Chem. 259, 6056-6058, 1984.

2. Zweier, J. L.: Iron-mediated formation of an oxidized adriamycin free radical. Biochim Biophys Acta, 839, 209-213, 1985.

3. Gianni, L, Zweier, J. L., Levy, A, and Meyers, C. E.: Characterization of the cycle of iron-mediated electron transfer from adriamycin to molecular oxygen. J. Biol. Chem. 260, 6820-6826, 1985.

4. Myers, C. E., Zweier, J., Gianni, L., Batist, G., Sinha, B., Muindi, J., Klecker, R., and Yeh, G.: The mechanism of adriamycin tumor cell kill. Preceedings of the UCLA Symposium - Leukemia Meetings, 1985, in press.

5. Zweier, J. L., and Weisfeldt, M. L.: Direct observation of free radical production in the post ischemic heart. Clinical Research, 33, 240A, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01452-02 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Time Resolved Fluorescence Spectroscopy

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	J. R. Knutson	Sr. Staff Fellow	LTD:NHLBI
Others	R. F. Chen	Sr. Investigator	LTD:NHLBI
	C. H. Scott	Biologist	LTD:NHLBI
	P. Chaudhuri	Student	LTD:NHLBI

COOPERATING UNITS *(if any)*

NHLBI: LB (A. Ginsburg, P. Kasprzyk), Biology Dept., JHU (L. Brand, L. Davenport, J.M. Beechem, B. Packard, M. Edidin, P. Neyroz, D. Walbridge) NHLBI:CM (R. Kincaid), Geoergetown U. (P. Hensley) and GWU (O. Alabaster)

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.0	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A new time-resolved fluorescence spectrophotometer was assembled, tested and modified. It was converted to a "T" format for simultaneous acquisition of polarization and decay data. While many applications planned for this instrument require interfacing and computing links still under construction, it has already proved valuable in the analysis of protein fluorescence. It produces data that, when combined with "global analysis" programs (see references), provides closer decay resolutions than previous instruments. Previously undisclosed components were found in proteins such as HLADH and BSA. The enzyme glutamine synthetase (GS) from the laboaratory of Ann Ginsburg was studied. It was found to undergo a specific conformational response to inactivation in a ternary complex with cofactor ADP. Very closely related analogs of ADP were, in contrast, unable to effect the same tryptophyl site perturbation.

Recently, the instrument was used to measure a sixfold slowing of Brownian rotations that accompanies the binding of mellitin to calmodulin. The polarization decay capabilities of the instrument were also used to verify the rigid binding of a new bifunctional fluorescent label within IgG molecules.

More recently, the subnanosecond resolution of the instrument was employed to characterize the exclusively excited-state protonation of serotonin. A theoretical framework was devised that should apply to other trp derivatives. In addition, a variety of smaller projects were begun or participated in:

- autocorrelation methods for decay instruments.
- excited state deprotonation mechanism of popular FACS pH indicator "DCH".
- Shape (axial ratio) of HLADH from excitation tuning (with globals)
- mechanism of the self quenching of dyes inside liposomes.

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Project Description

Objectives:

We wish to develop instrumental approaches to sharpen and clarify the time resolution of fluorescent signals from proteins and membrane structures. This will help us to untangle the complex emissions that biological systems display. Once these systems are broken down into components, it becomes possible to associate spectral features with reporting groups that represent particular sites and/or conformations. Binding events (and related biological events) can thereafter be examined as they perturb associated spectra. Thus, one can trace the dynamic effects of each event to specific sites in the macromolecule. Fluorescence spectra and decays are exquisitely sensitive indicators of structure and dynamics. The only bar to their effective use is the difficult task of "untangling" complex, mixed signals. The state-of-the-art instrument we have developed will, when combined with our recent data analysis schemes, greatly facilitate the recovery of multiple spectra from complex systems. These resolved components will help us understand the basic function of dynamic structures in proteins and lipid bilayer membranes.

Major Findings:

A new time-resolved fluorescence spectrophotometer was assembled, tested and modified. The design of this instrument has evolved in response to recent changes in both computer and general electronic technology. For example, new software incorporating "global analysis" principles (Knutson, et. al., 1983, 1985, Beechem et al. 1983, 1984, 1985) make a T-format instrument tenable for the first time. Thus, we have converted the sample chamber/detector configuration to exploit this possibility. The true T operation will soon become available (upon installation of new electronic signal routing modules).

The new instrument was used to examine a variety of protein systems containing intrinsic fluorophores (eg., trp or tyr residues) The enzyme glutamine synthetase (GS) was studied in collaboration with the laboratory of A. Ginsburg. Using the DAS technique (Knutson et. al, 1982), we resolved two different spectral responses of tryptophyl residues (2/subunit) to the formation of specific enzyme-cofactor-inhibitor complexes. It appears that very minor chemical modifications to the cofactor strongly perturb the environment of one of the residues (conversely, one residue seems little affected, except for a minor long range quenching). In particular, the sensitive residue appears to move to a "buried" location upon formation of the ADP complex, but is "exposed" in the corresponding native dodecamer. It is almost as "exposed" in complexes with ADP analogs bearing sulfur at A6 or A8 positions. The "buried" vs "exposed" condition is seen in decay time, yield and spectral center. A higher resolution DAS process will be applied

to this and other systems upon completion of our current automation/interfacing changes. For example, BSA and HLADH have both been found to contain spectral components that were not resolved by other instruments. The BSA molecule appears to place Trps in two different environments. One species (likely one of the two residues) is in a hydrophobic regime. The other begins in a similar mileiu, but rapidly forms an excited state complex that is redshifted (and slowly decaying). We were able to examine the traces of the parent state that disappear within a nanosecond. This is directly attributable to the linkage of our instrument to global analysis procedures (Knutson et al, 1985). We continue to develop those methods, in collaboration with the Brand Laboratory (Beechem et al 1983, 1984, 1985; Knutson et al 1983, 1984, 1985).

The instrument has also been applied to a variety of other problems. The rigid and specific labeling of IgG molecules by a new dye ("Crabescein": B. Packard and M. Edidin of JHU) was evaluated using ultraviolet pulses to generate (negative) polarization of green luminescence. A similar approach was used to examine the binding of mellitin (R. F. Chen, C. H. Scott, R. Kincaid) to calmodulin. Similarly, tunable ultraviolet was used to determine the axial ratio of HLADH dimers (Beechem et al, 1985).

Recently, the excited state reaction responsible for an acid-induced visible fluorescence of serotonin (and other 5-alkyloxy indoles, see Chen, 1967) has been characterized. Extensions of the standard (two-state) theory were developed to account for the parasitic and static quenchings that occur in this system. Other excited state protonation/deprotonation systems were studied (DCH:O. Alabaster; equilenin, dehydroequilenin: L. Davenport) via steady state measurements.

Significance to Biomedical Research and the Program of the Institute:

Fluorometry is an increasingly important tool in biochemisty. This is largely due to the exceptional sensitivity to surroundings that fluorophores exhibit, along with the variety of signals made available by these probes. Improved fluorescence technology translates directly into improved knowledge of protein and membrane function. Currently, the major limitation to biophysical fluorometry is complexity, and this complexity can be solved with improved instruments and their accompanying data analysis methods.

Proposed Course:

We will continue to develop our time-resolved fluorometer with the goal of providing the utmost attainable accuracy and power. We will evaluate model systems including multisubunit protein complexes and bilayer membranes that contain multiple domains. These studies will be conducted using the most recent advances in luminescence data analysis. In all, we will seek to produce the premier fluorescence lifetime and anisotropy instrument, a tool

that will reveal sharper detail about macromolecular structure and function.

In particular, we will reduce the width in timing response of the system, speed the collection of simultaneous decay curves, establish the ability to scan (rather than select) excitation, and computerize the data surface scanning process.

At the same time, we will "wrap up" and publish examples of the new kinds of structural information provided by this instrument. Whenever feasible, we will also prototype some of our fluorometric ideas that more radically depart from current technology (eg, autocorrelation as/of detectors, transverse gating of photomultipliers, dielectric resonant anisotropy).

Publications:

"Associated Spectra and the Multidimensional Nature of Fluorescence Spectroscopy" J. R. Knutson, L. Davenport, J. M. Beechem, D. G. Walbridge and L. Brand. in "Excited State Probes in Biology and Medicine" (NATO ASI, Eds. Szabo, A.G., and Masotti, L., Plenum, in press).

"Time-Resolved Fluorescence Spectroscopy: Some Applications of Associative Behavior to Studies of Proteins and Membranes" L. Brand, J. R. Knutson, L. Davenport, J. M. Beechem, R. E. Dale, A. A. Kowalczyk and D. G. Walbridge. In "Spectroscopy and the Dynamics of Molecular Biological Systems" pp 259-305 (BBS), Eds. Bayley, P. M. and R. E. Dale, Academic Press, 1985.

"Decay Associated Spectra (DAS) provide site specific information about the binding of small molecules to proteins" J. R. Knutson, D. G. Walbridge and L. Brand, "Nanosecond Fluorescence Decay and Protein Kinetics: Rapid Data Accumulation and Pulse Fluorometry" D. G. Walbridge, J. R. Knutson and L. Brand, both in: Proceedings, Excited State Probes in Biology and Medicine (NATO ASI) Acireale, 1984.

"Excited State Proton Transfer Mechanisms of Equilenin and Dihydroequilenin: Effect of Liposome Binding" L. Davenport, J. R. Knutson and L. Brand. In Proceedings, Int'l Conference on the Application of Fluorescence in the Biomedical Sciences (CFR, Pittsburgh, 1985).

"Protein Hydrodynamics from Global Analysis of Anisotropy Decays" J. M. Beechem, J. R. Knutson, and L. Brand, Biophys J. 47: 411A (1985).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 01455-01

PERIOD COVERED: October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Development of Foam Countercurrent Chromatography

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I. Y. Ito Senior Investigator LTD:NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A novel method of performing foam separation has been developed. The method uses a gas-liquid dual countercurrent flow through a helical column subjected to a particular type of synchronous planetary motion. Samples introduced at the middle portion of the column, in either batch or continuous mode, are separated according to the foam affinity. Any material having an affinity to the foam is quickly carried with the foaming stream and eluted through one end of the column whereas other materials are carried with the liquid stream in the opposite direction and eluted out through the other end of the column. Capability of this foam countercurrent chromatographic method is demonstrated on separations of rhodamine B and Evans blue with an anionic surfactant, SDS, as a collector of rhodamine B. Successful preliminary separation of protein samples, BSA and sheep hemoglobin, indicates that the present method may be effectively applied to separation and purification of various biological samples such as enzymes, membrane receptors, etc.

PHS 6040 (Rev 1/84) GPO 904-917

Project Description

Objectives: Development of a novel method of foam separation using dual countercurrent system.

Methods Employed and Major Findings:

Principle:

When a coiled tube is subjected to a particular type of planetary motion, two immiscible solvent phases present in the coil are unilaterally distributed along the length of the coil, one phase (head phase) entirely occupying the head side and the other phase (tail phase) the tail side of the coil. Here, the head and tail of the coil are defined on the basis of the Archimedean screw effect - all objects in the coil tend to move toward the head of the coil. The above unilateral phase distribution allows a true or dual countercurrent flow of the two phases through the coil upon simultaneous introduction of the head phase through the tail and the tail phase through the head of the coil This dual countercurrent operation requires two flow channels at each end of the coil, one for feeding and the other for collecting the effluent, while the sample feed port may be made at the middle portion of the coil.

The dual countercurrent system above described can be applied to foam separation by using gas as a tail phase. When the liquid phase (head phase) contains a surface-active substance, countercurrent process produces foam which moves quickly toward the tail of the coil. Consequently, samples introduced at the middle portion of the coil through the sample feed port are continuously separated according to the foam affinity. Any material having foam affinity is carried with the foaming stream and eluted through the tail of the coil while other materials are carried with the liquid stream in the opposite direction and eluted out through the head of the coil. When the samples have foam-producing capability as detergents, the separation may be performed without surfactant (collector) in the feeding liquid phase.

Apparatus:

The experiments were performed with a combined horizontal flow-through coil planet centrifuge which holds a pair of column holders symmetrically at a distance of 20 cm from the centrifuge axis. Each holder produces a different mode of synchronous planetary motion. The pulley-driven holder synchronously counterrotates around its own axis while revolving around the central axis of the apparatus. The gear-driven holder gives rotation and revolution at the same angular velocity in the same direction. Both types of planetary motions prevent twisting the flow tubes and therefore eliminate the need for the conventional rotary seals. Preliminary experiments have shown that the pulley-driven holder failed to establish unilateral phase distribution in the coil while the gear-driven holder produced an ideal unilateral phase distribution in the coil with a proper diameter of the coil holder. Therefore, the gear-driven holder was exclusively used in the present experiments.

The column was prepared from a 10 m long, 2.6 mm i.d. PTFE tube by winding it coaxially onto the holder of 5 inch diameter making two coiled layers. Using Kel-F (polytrifluoromonochloroethylene) 3-way adaptors, two flow channels were connected to each end and one flow channel at the middle portion of the coil for sample feeding. The total capacity of the coiled column measured about 50 ml.

Elution Modes of the Apparatus:

The capability of the apparatus was evaluated on foam separation of rhodamine B (and Evans blue) using a sodium dodecyl sulfate (SDS) solution. The experiments were performed in the following three different elution modes.

1. Continuous enrichment and stripping without using the sample feed line: a large volume of the sample solution containing small amounts of material is pumped through the liquid feed line (tail) while the nitrogen gas is introduced through the gas feed line (head) at constant pressure of 80 psi. The enriched foam is continuously collected through the foam collection line (tail) and the stripped liquid, through the liquid collection line (head).

2. Batch separation with sample injection through the sample feed line: Continuous countercurrent streams of gas and liquid phases are introduced along the length of the column by pumping the liquid phase through the liquid feed line (tail) under N_2 gas pressure of 80 psi applied through the gas feed line (head). After the steady state hydrodynamic equilibrium is established, a small volume of the sample solution is locally injected through the sample feed line. Foam and liquid eluted through the respective collection lines are separately fractionated into a series of test tubes at suitable intervals.

3. Continuous separation by continuous sample feeding through the sample feed port: The steady state equilibrium of the gas-liquid countercurrent flow is first established in the column as in the batch separation experiment described above. Then, the sample solution is continuously introduced through the sample feed line. Foam from the foam collection line and the effluent form the liquid collection line are each separately collected into a graduated cylinder for later analysis.

Experimental Results:

1. Continuous Enrichment and Stripping

This experiment was performed to demonstrate the capability of the method to concentrate and/or eliminate a minute amount of material present in a large volume of sample solution. The sample solution containing rhodamine B at a 10^{-6} M concentration and SDS at 10^{-3} M as a collection was introduced through the liquid feed line at 214 ml/h against N_2 flow through the gas feed line at 80 psi, while the apparatus was run at 500 rpm. The sample feed line was not used in this experiment. The liquid collection rate was adjusted at a level slightly below the liquid feed rate so that the foam collection rate became as small as several hundred microliters per hour which yielded the foam highly enriched with rhodamine B. After 1 liter of the sample solution was

eluted, the liquid collection rate was adjusted at a level slightly below the liquid feed rate so that the foam collection rate became as small as several hundred microliters per hour which yielded the foam highly enriched with rhodamine B. After 1 liter of the sample solution was eluted, the liquid collection line was closed to elute rhodamine B remaining in the column through the foam collection line. The stripped liquid collected through the liquid collection line was fluorometrically analyzed to determine the concentration of rhodamine B. The results showed that the dye concentration in the stripped solution was 1.3×10^{-9} M while over 99% of rhodamine B was recovered through the foam collection line within a 2 ml volume resulting in over 500-fold enrichment.

Batch Separation with Sample Injection through Sample Feed Line

This experiment was initiated by establishing a liquid-gas countercurrent flow equilibrium through the coiled column. At the revolutional speed of 500 rpm a surfactant solution containing SDS at 10^{-3} M was pumped through the liquid feed line while the N_2 gas flow was introduced through the gas feed line at 80 psi. After the hydrodynamic equilibrium was reached, 0.5 ml of sample solution containing rhodamine B and Evans blue each at 5×10^{-4} M was injected through the sample feed line. The needle valve on the liquid collection line was adjusted to make a 1:3 volume ratio between the foam and liquid fractions. Effluents from both collection lines were separately fractionated into a series of test tubes at 30 second intervals. The concentration of each dye in the fractions was spectrophotometrically determined using 556 nm for rhodamine B and 620 nm for Evans blue.

The chromatogram obtained through the foam collection line showed a sharp single peak entirely consisting of rhodamine B with the peak maximum at one minute after sample injection. The chromatogram obtained through the liquid collection line showed a broad symmetrical peak of Evans blue with the peak maximum at 2.75 minutes after sample injection. These results were quite reproducible and injection of the single component produced the similar peak through the respective collection line. The volume of the liquid phase present in the column under a steady state hydrodynamic equilibrium in these experiments ranged between 4 and 5 ml which amounted to approximately 10% of the total column capacity.

3. Continuous Separation by Continuous Sample Feeding through the Sample Feed Line

Rapid and clean separation of the two dyes in the batch separation method described above indicated the feasibility of continuous separation by steadily feeding the sample mixture at a proper rate. Under otherwise identical experimental conditions used in the batch separation, the sample solution was continuously introduced through the sample feed line at various flow rates. The satisfactory separations were obtained at sample feed rates of 0.36 ml/min or less, which separated each sample at the maximum rate of 1.8×10^{-7} mol/min. The application of higher flow rates resulted in initial accumulation of rhodamine B in the column which was later followed by elution of rhodamine B through the liquid collection line.

The experiments were continued to study the effects of SDS concentration on the hydrodynamic behavior of rhodamine B in the foam separation column under a high sample feed rate of 3.8×10^{-7} mol/min. The results showed that at a high SDS concentration of 10^{-2} M, the rhodamine B exhibited little affinity to the foam and mostly eluted through the liquid collection line. As the SDS concentration was decreased, the dye rapidly developed the foam affinity and, at 10^{-3} to 2×10^{-3}M SDS concentrations, over 90% of the dye was collected through the foam collection line while the liquid collection line eluted clear liquid almost free of rhodamine B. Further decrease of the SDS concentration resulted in a decreased foam recovery rate of rhodamine B causing the retention within the separation column. If the sample feeding is stopped at this stage, elution of the dye through the foam collection line continues at the same rate until the retained dye is completely recovered. If the sample feeding is continued, the dye continuously accumulates in the column and finally appears through the liquid collection line. The results clearly indicate that the foam recovery rate of rhodamine B is largely governed by the SDS concentration which yields the highest recovery rate at 10^{-3} M to 2×10^{-3} M under the present experimental condition.

4. Preliminary Experiments for Protein Separation

The present method has been applied to the separation of proteins without the use of a surfactant collector. As is well known, exposure of proteins such as BSA to a gas-liquid interface may cause denaturation to alter the physiological function of the molecule. The preliminary studies were conducted to test vulnerability and foam-producing capacity of proteins with the present system by injecting the sample solution into the running column through the sample feed line. Several kinds of proteins including BSA, human and sheep hemoglobin, and ovalbumin were examined. Among these only BSA showed an active foam-producing ability and was collected through the foam collection line whereas other proteins were mostly eluted through the liquid collection line without any visible evidence of denaturation. BSA fractions eluted through the foam collection line showed various degree of turbidity apparently due to denaturation of the molecule. Further experiments revealed that the intensity of turbidity highly depended upon the composition of the applied liquid phase. The use of salt-free distilled water or dilute acid solution caused most intensive turbidity. Addition of a surfactant to the liquid phase decreased the degree of turbidity but at the same time lowered the foam recovery rate of BSA. Sodium phosphate solution of slightly alkaline pH (7.2 - 8.9) at a relatively high ionic strength (0.2 - 0.5 M) produced minimum turbidity with a high BSA recovery of over 90% through the foam collection line.

The preliminary experiments for the batch separation of BSA and sheep hemoglobin have been successfully performed with a liquid phase composed of 0.2M dibasic sodium phosphate solution (pH 8.9) under the standard experimental conditions previously applied to the dye separation. BSA with a foam-producing capability was quickly eluted through the foam collection line within 6 minutes while sheep hemoglobin was entirely recovered through the liquid collection line in about 10 minutes.

Significance to Biomedical Research and the Program of the Institute:

Although the foam separation method was known to be a versatile separation method covering a broad range of samples from small ions to macromolecules and particles, the technology has remained primitive and inefficient, limiting its application in the research laboratories. The preliminary results of the foam countercurrent chromatography suggest that this new method would drastically improve both separation times and efficiencies of the conventional methods to become an essential tool for scientific research in the near future. Among various applications, the preliminary results of protein separation indicate that the present method may be applied to an affinity separation of biological samples. For example, a long hydrocarbon chain is attached to a substrate or inhibitor molecule of the aimed enzyme to form a hydrophobic terminal so that the derived molecule acquires a foam affinity to carry the enzyme. The enzyme prebound to such collector molecules may be efficiently concentrated and isolated with foam in a short period of time. Achievement of the above goal, would make a great contribution to a broad field of biological sciences including protein chemistry, cell physiology and genetic engineering.

Proposed Course:

1. Refinement of the apparatus and the column for foam countercurrent chromatography.

2. Systematical studies on distribution of various samples in the column using a series of surfactants.

3. Exploration of various applications including foam affinity separation of macromolecules and cell particulates.

Publication:

Ito, Y.: Foam Countercurrent Chromatography Based on Dual Countercurrent System, J. Liq. Chromatogr., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01456-01 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of a Pulsed Light Source, Single Photon

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principel Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	R. L. Berger	Chief, Biophysical Instrum Section	LTD:NHLBI
	W. Friauf	Chief, Section Elect. Engineering	BEIB:DRS
	C. Reimer	Chief, Section Immunochemistry	DHR:CDC

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Technical Development

SECTION

Biophysical Instrumentation Section

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	.5	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ability to detect minute amounts of protein is of interest both in protein chemistry and immunology. A possible replacement for radioimmuno assay (RIA) is the tagging of the protein by a caged molecule of Europium. By taking advantage of the very long fluorescence lifetime of Europium (500 microseconds) extreme sensitivities are possible. A pulsed nitrogen laser operating at 337 nm at 15 pps and power output of 200 microjoules been used with a ½ inch end on PET photomultiplier tube to examine a simple Europium tagged system.

926

Project Description:

Objectives:

The objective of this project is to develop a protein fluorescence detection system which will have a higher sensitivity than that presently available with RIA. The design goal is 10^{-16} M.

Methods Employed:

The Method of pulsed light excitation of Europium and single photon counting will be exploited for this instrument development. Eu has a 500 μs fluorescence half-life so that by shutting off the photomultiplier for the first 50 μs, after a 17 ns laser pulse, one then looks at a black box. Detection sensitivity is then only limited by system noise.

Major Findings:

The instrument development so far has consisted of a preliminary system tested to a sensitivity of 10^{-13} M to prove the over all concept. A redesigned system with an improved nitrogen laser has now been built and is presently being tested.

Significance to Biomedical Research and the Program of the Institute:

The ability to rapidly determine protein levels in serum, to 10^{-16} M, would greatly extend the range and sensitivity of antigen-antibody reactions. This has great advantages for early detection of a whole host of viral diseases, such as AIDS, etc.

Proposed Course:

A complete package will be developed and tested with delivery to the CDC expected in July. A second unit will be built for further studies here at NIH.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01457-01 LTD

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Instrumentation for Intracellular Injection of DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I. R. L. Bowman Chief, Lab. Technical Development LTD:NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
½	½	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The introduction of a few molecules of DNA into the pronucleus of mouse oocytes yields enough viable offspring to make the procedure practical but limitation on the number of oocytes that can be injected by this method and the decreasing yield with smaller cells indicates the desirability of better instrumentation. The alternate method of gene insertion using viruses is more successful but has undesirable aspects related to the use of the virus.

Automation and high through put can be accomplished by either high speed serial injection or a slower parallel method. In the former delivery of single cells to one or more injection devices that obviates the handling of microinjections under the microscope could be accomplished by flowing single cells to a "pocket" that holds the cell while the injection is accomplished and then returns the cell to the stream to be collected and cultured.

We have elected to explore the former serial procedure to demonstrate the "grab and stab" idea first because the apparatus can be assembled here and tested to indicate the need and potential for success of the plate technique which would be less accessible and much more expensive.

A micro "glass" lathe has been constructed that holds the work between synchronously rotating chuck that can be separated or opposed while the work is being rotated in a micro flame mounted on a micromanipulator. A steric microscope pounds up to 90X magnification. A flame method is required because I believe the high melting point and short working range will permit more minute structures to be worked. Some preliminary trials have identified problems that are being corrected but the basic idea of the advantages of quartz are essentially confirmed.

928

Project Description

To provide instrumentation that can inject DNA into a substantial number of cells that will go on to form hybreds.

Methods Employed:

A micro glass lathe is being developed that will permit quartz to be worked in a micro flame. A "glass" lathe is special in that both ends of the glass are supported and rotate synchronously while a flame is applied to soften the work. A means of blowing air inside the tube is provided by a rotating joint and a means of moving the spindles together or apart while rotating is used to draw down or make end seals while the flame keeps the work pliable.

A very rigid structure was fabricated using oversize linear ball bearings that holds the spindles in alignment but permits the spindles to move together or apart while synchronous motion is imparted through a system of gears that slide along a long motor driven spline.

The maximum size that can be held in the spindle chuck is 1 mm and this is accomplished by utilizing the mass produced pencil lead chucks from Pentil brand pencils. These are available down to 0.2 mm but so far only the .9 and .5 mm chucks have been obtained. Tests on the .9 mm chucks have had marginal success due to the short length of the jaws. A tandum arrangement might correct this but not yet accomplished.

micro flames to heat very small sections of the work require a special construction flame that I have used for some time that burns house gas in a pure oxygen atmosphere produced by excess flow of oxygen around a small central gas jet. The excess oxygen flow imparts a cooling sheath of gas that cools the work around the central hot core thus making it possible to heat a very restricted portion of the work while the oxygen excess cools adjacent material. This flame has been used as well as pure O_2 + H_2 provided by electrolysis of H_2O. This stoichiometric mixture burned at the tip of a #27 hypodermic needle is a simpler system and has to be compared with and without addition of methanol to the mixture to determine which is the most useful.

Major Findings:

So far the work has concentrated on developing a satisfactory method of working the quartz. The principle problems have turned out to be effective methods of holding the stock concentric within the few microns needed. There are no satisfactory means of holding stock in the 40-50 micron size except by sealing into drawn down 1 mm tubes.

Experiments have shown that this is relatively easily accomplished and it may be practical to use a springy fit 1 mm chuck and do all work with sealed in or drawn material from 1 mm stock.

Significance to Biomedical Research and the Program of the Institute:

Somatic gene cell therapy techniques and the application in humans should result in at least a partial correction of a number of genetic disorders. Instrumentation of the tehcniques of DNA injection may be of great importance in facilitating the studies necessary to establish the safety and limitation of the technique.

Proposed Course:

To perfect the system until micro injection and selection techniques demonstrate the feasibility of this approach. Design of flow systems and automation to be delayed until we show the apparatus can be constructed. No action on the parallel injection system is proposed at this time.

Publications:

None

Annual Report of the Pulmonary Branch
National Heart, Lung and Blood Institute
October 1, 1984 through September 30, 1985

The research of the Pulmonary Branch centers on diseases of the alveolar structures, the site in the body in which gas exchange takes place between the air and blood. Three categories of common diseases are investigated, including: (1) disorders characterized by fibrosis of the lung parenchyma; (2) disorders characterized by the accumulation of T-lymphocytes in the lower respiratory tract; and (3) disorders characterized by destruction of the alveolar walls. All three categories represent chronic inflammatory disorders in which the inflammation is compartmentalized to the lower respiratory tract and in which the inflammation causes the changes in the lung parenchyma that defines the clinical presentation of each disease.

The disorders characterized by progressive fibrosis of the lung parenchyma represent a large subgroup of the interstitial lung disorders, chronic disorders in which derangements of the alveolar wall are accompanied by "fibrosis", an accumulation of mesenchymal cells and the collagenous matrix produced by these cells. In general, the inflammation of these disorders is dominated by alveolar macrophages, and to a lesser extent, neutrophils and/or eosinophils. Example of these fibrotic disorders include idiopathic pulmonary fibrosis and asbestosis.

The disorders characterized by the accumulation of T-lymphocytes in the lower respiratory tract are also a subgroup of the interstitial lung disorders. However, although occasionally associated with progressive fibrosis of the lung parenchyma late in their course, these disorders are universally characterized by large numbers of T-lymphocytes that dominate the inflammation and cause dysfunction by their presence, which distorts the alveolar, bronchial, and vascular walls, thus modifying the intimate relationship between air and blood. Examples of the T-lymphocytes disorders include sarcoidosis, berylliosis and hypersensitivity pneumonitis.

The disorders characterized by destruction of the alveolar walls are commonly called emphysema. Of the $2x10^6$ individuals in the USA with emphysema, approximately 98% acquire the disease, usually on the basis of cigarette smoking, while 2% have an inherited disorder called alpha-1-antitrypsin deficiency. All forms of emphysema are characterized by a dissolution of the lung parenchyma. The inflammation that causes these changes is dominated by alveolar macrophages together with smaller numbers of neutrophils.

The inflammation of all of these disorders can be evaluated by bronchoalveolar lavage, a technique that permits direct access to the epithelial surface of the lower respiratory tract where inflammatory cells and relevant molecules in the epithelial lining fluid can be easily and repetitively sampled. To accomplish bronchoalveolar lavage, local anesthesia is administered to the upper respiratory tract and a fiberoptic bronchoscope is gently wedged into a distal bronchus. Sterile saline, usually

5, 20 ml aliquots in 1 to 3 sites, is infused into the bronchoscope and then suctioned back, thus sampling the epithelial lining fluid of the lower respiratory tract. Over the past decade, this procedure has been used by the Pulmonary Branch to evaluate several thousand individuals.

The current concepts of the mechanisms of pulmonary fibrosis hold that the accumulation of fibroblast and fibroblast products result from two general processes. First, there must be damage to the alveolar wall; in most cases this is mediated by products of inflammatory cells, primarily neutrophils and/or eosinophils but including macrophages. Second, that there is enhanced proliferation of mesenchymal cells in the alveolar walls driven by growth signals released by alveolar macrophages.

The bulk of the injury caused by neutrophils, eosinophils and macrophages result from the ability of these cells to release toxic oxidant radicals such as superoxide, hydrogen peroxide and hydroxyl radical. In addition, neutrophils, and to a lesser extent eosinophils and young macrophages, can release peroxidases that, in the presence of hydroxyl radical and a halide such as the chloride anion, produce the toxic hypohalide radical. Recent studies have demonstrated that the lung has protection on its epithelial surface to prevent oxidant mediated injury. Included among these antioxidants is glutathione, a tripeptide that is an effective inhibitor of hydrogen peroxide. The glutathione levels on the epithelial surface of the human lower respiratory tract are seventy times that in plasma. Interestingly, the glutathione levels in the epithelial lining fluid of the lungs of cigarette smokers is higher than that of normal while the glutathione levels in patients with idiopathic pulmonary fibrosis are lower. While the mechanisms of the accumulation of glutathione in the lower respiratory tract are not known, it is clear that alveolar macrophages release glutathione as do some parenchymal cells. In addition to glutathione, there are other small molecular weight antioxidants in the epithelial lining fluid including vitamin E. Furthermore, there are large molecular weight protein antioxidants including catalase. Although catalase is an intracellular antioxidant, it is found in large amounts on the epithelial surface of the lower respiratory tract, presumably released by epithelial cells during their process of normal cell turnover. Independent of the mechanisms for the accumulation of catalase, it is a very effective antioxidant against hydrogen peroxide and thus affords significant protection to the lower respiratory tract.

Although eosinophils are classically considered to be a "protective" cell for inflammatory and immune processes, there is increasing evidence that the eosinophil can effect damage to normal tissues. To evaluate this concept in the lower respiratory tract in man, it is necessary to find a "pure" eosinophilic inflammatory process. To accomplish this, in conjunction with the Laboratory of Clinical Investigation of the National Institute of Allergy and Infectious Diseases and the Tuberculosis Research Center, Madras, India, and the Indian Council for Medical Research, under the auspices of the Indo-USA joint agreement on technology, members of the Pulmonary Branch evaluated patients with acute tropical pulmonary eosinophilia in Madras, India. These individuals have a pure eosinophil inflammatory process in the lower respiratory tract that includes up to 70% of the total inflammatory cells being eosinophils (normal less than 1%). When treated with diethylcarbamezine, the eosinophil inflammatory process is suppressed and the patients improve. This preliminary study was followed up with the study of patients that had been previously treated with diethylcarbamezine and were reevaluated six months to two years later. Strikingly, they continued to have a low level of an eosinophil inflammatory process in the lower tract with concomitant

evidence of a mild fibrotic lung disease. Evaluation of the inflammatory cells recovered by bronchoalveolar lavage revealed that they were releasing increased amounts of superoxide and hydrogen peroxide (compared to normal inflammatory cells). Furthermore, when treated with corticosteroids, the level of oxidant production by these cells diminished.

The inorganic dust disorders including asbestosis, silicosis, and coal worker pneumoconiosis are all characterized by damage to the parenchymal cells of the lower respiratory tract. Evaluation of the inflammatory cells of nonsmoking individuals exposed for long periods to these inorganic dusts demonstrates that, in many cases, the alveolar macrophages are releasing superoxide and hydrogen peroxide. In this context, one way to approach the therapy of these disorders is to augment the antioxidant screen of the lower respiratory tract or to suppress the production of these oxidants by the inflammatory cells that have accumulated.

All of the fibrotic lung diseases are characterized by an increase in the number of alveolar macrophages in the lower respiratory tract. The classic concept of accumulation of mononuclear phagocytes in tissues suggests that there is an influx of monocytes to the tissues likely called in by chemotactic factors. In individuals exposed to asbestos, evaluation of alveolar macrophages recovered by bronchoalveolar lavage revealed that a significant number of the macrophages on the epithelial surface of the lung express monocyte lineage antigens, consistent with the concept that they represented newly recruited monocytes. Furthermore, evaluation of alveolar macrophages from individuals with a variety of fibrotic lung diseases, including asbestos exposure, has demonstrated that a proportion of these macrophages are proliferating, suggesting that enhanced replication in situ also contributes to the process of macrophage accumulation. In this context, it is known that for all mammalian cells to proliferate they require iron. The major, if not the only, mechanism by which iron gains access to mammalian cells is through the carrier protein transferrin. Interestingly, while blood monocytes, the precursor of the alveolar macrophage, do not have transferrin receptors, alveolar macrophages do. Characterization of human alveolar macrophage transferrin receptors by monoclonal antibodies, receptor-ligand binding studies, and evaluation of transferrin receptor mRNA levels has confirmed this concept. Thus, although the mediators that stimulate alveolar macrophage to proliferate in these diseases are not known, the alveolar macrophages do have at least one receptor which is likely necessary to allow it to proliferate.

Tissue fibrosis results, in part, from an interaction between growth regulatory molecules released by mononuclear phagocytes and the fibroblast target cells. In the chronic interstitial lung disorders, alveolar macrophages are known to be spontaneously releasing increased amounts of two growth factors for fibroblasts, fibronectin and alveolar macrophage derived growth factor (AMDGF), that together stimulate nonreplicating lung fibroblasts to divide. In addition to these two primary growth signals, alveolar macrophages also release other mediators that may have the potential role in modulating lung fibroblast replication. However, recent evaluation of several of these mediators, including interferon-γ and interleukin-1, suggests they have little (interleukin-1) or no effect (interferon-γ). Prostaglandin E_2, another macrophage product, suppresses fibroblast replication in a dose-dependent manner.

Recent studies have shed additional light on the genes involved in growth factor production by mononuclear phagocytes. These studies have shown that activated human blood monocytes release a mediator that is capable of attracting smooth

muscle cells and cooperating with other mediators to stimulate fibroblasts to divide. Purification of this mediator demonstrated that its chromatographic properties and chemical stability had a close similarity to platelet derived growth factor (PDGF). Consistent with this concept, the partially purified monocyte mediator displaced ^{125}I-labelled PDGF from its receptor on BALB/c-3T3 cells and immunoprecipitated with antibodies directed against PGDF. Strikingly, conditions which stimulated monocytes to release this PGDF-like molecule also stimulated the monocytes to express the gene coding for the c-sis proto-oncogene, a gene on chromosome 22 known to code for one of the PDGF chains. In contrast, resting monocytes did not secrete the PDGF-like molecule nor express detectable amounts of c-sis mRNA. These observations are consistent with the concept that expression of the c-sis proto-oncogene may play a role in enabling mononuclear phagocytes to modulate the local accumulation of mesenchymal elements and have implications for the process of normal wound healing as well as for the pathogenesis of several common chronic human disorders. In the lung, studies have demonstrated that alveolar macrophages express the c-sis proto-oncogene and are capable, when stimulated, of releasing a PDGF-like molecule. Furthermore, in idiopathic pulmonary fibrosis, alveolar macrophages are releasing markedly increased amounts of these PDGF-like molecules compared to normal resting alveolar macrophages. Thus, while the role of this PDGF-like molecule in the pathogenesis of the fibrotic lung diseases is not clear, it does appear that expression of the c-sis proto-oncogene correlates with the release of a functional molecule by these cells.

To gain insight into the early mechanisms of fibrosis in idiopathic pulmonary fibrosis, three families with familial pulmonary fibrosis have been evaluated in detail. The disease familial pulmonary fibrosis shares with idiopathic pulmonary fibrosis a characteristic pattern of alveolar inflammation featuring neutrophil accumulation and macrophage activation. Strikingly, despite being clinically normal in every respect, about half of the first degree relatives of individuals with familial pulmonary fibrosis have clear evidence of a similar pattern of alveolar inflammation despite the fact that they have no evidence of structural damage to their lower respiratory tract. This observation raises the intriguing issue of whether these individuals are at risk of developing disease in the future.

In collaboration with INSERM, Paris, and the Pathology Branch, NHLBI, a recent study of several hundred biopsies of patients with interstitial lung disease has demonstrated that the classic view of the "interstitial" nature of the fibrosis in these diseases is too simplified. It is apparent from the ultrastructural evaluation of these biopsies that intraalveolar fibrosis also plays an important role and that incorporation of this intraalveolar process into the wall of the alveoli contributes to the expansion of the thickness of the alveolar wall with fibroblast and fibroblast products.

In the context of the mechanisms of pulmonary fibrosis discussed above, it is apparent that therapy should be directed to suppressing the inflammatory cells that cause the damage to the alveolar walls and to the inflammatory cells (i.e., macrophages) that are driving the fibroblasts to proliferate. In this regard, a study was carried out comparing corticosteroids to cyclophosphamide for their ability to suppress the neutrophil component of the alveolitis in idiopathic pulmonary fibrosis. On the average, glucocorticoids had little effect while cyclophosphamide markedly suppressed the numbers of neutrophils in the lung when cyclophosphamide was used alone or in combination with corticosteroids. Like their effect on the neutrophil component of the inflammation, glucocorticoids have

little effect on the release of fibronectin and AMDGF by alveolar macrophages of patients with fibrotic lung disease. This is despite the fact that alvoelar macrophages have glucocorticoid receptors on their surface. Interestingly, colchicine in doses that likely can be achieved in vivo will suppress the release of fibronectin and AMDGF, at least in part, by alveolar macrophages of these patients.

Pulmonary saracoidosis is a disorder of the lower respiratory tract characterized by chronic inflammation, granuloma formation and, in some individuals, parenchymal fibrosis. Together these processes derange the alveoli, airways and blood vessels, consequently impairing the ability of the lung to exchange gas in the normal fashion. As with the other interstitial lung disorders, it is recognized that the inflammation proceeds the other abnormalities that characterize this disorder. The inflammation of active pulmonary sarcoid is dominated by an accumulation of T-helper lymphocytes in the lung parenchyma. These T-cells are thought to play a central role in the pathogenesis of sarcoidosis in two ways. First, the accumulated T-cells distort the architecture of the parenchyma, thus altering the intimate relationships between air and blood. Second, the T-cell populations are activated and spontaneously releasing monocyte chemotactic factor and interferon gamma, mediators that recruit and activate mononuclear phagocytes, respectively, events that are early steps in the process of granuloma formation. In this context, an understanding of the pathogenesis of pulmonary sarcoidosis is intimately linked to the understanding of the process directing the accumulation of T-lymphocytes in the lower respiratory tract of individuals with active disease. Relevant to this question, prior work in this laboratory has demonstrated that the T-lymphocytes recovered from the lungs of these patients are spontaneously proliferating and spontaneously releasing interleukin-2 (IL-2), the T-cell growth factor. Thus, while the stimulus that initiates the process is unknown, the IL-2 releasing lung T-cells are thought to be responsible for maintaining the T-cell inflammation and thus maintaining the disease in an active state.

Recent studies have expanded upon this concept by determining that the IL-2 releasing cells in the lung are LEU3+ (helper-inducer) T-lymphocytes expressing HLA-DR antigens on their surface. Since this is the same subset of T-lymphocytes that likely release IL-2 in normal antigen driven T-cell proliferation, these observations lend credence to the concept that the T-cell inflammation of sarcoid is an exaggeration of the normal processes of T-cell activation. Consistent with this concept, studies utilizing an IL-2 cDNA probe have demonstrated that lung T-lymphocytes from individuals with active pulmonary sarcoidosis are spontaneously expressing the IL-2 gene while blood T-cells from the same individuals are not.

Since the IL-2 driven accumulation of helper T-lymphocytes in the lung plays a central role in the pathogenesis of sarcoidosis, it is reasonable to hypothesize if the interleukin-2 gene expression could be suppressed in lung T-cells, these cells would be prevented from accumulating in the lung parenchyma. To test this hypothesis, we prospectively evaluated the lung T-cells and lung function in a group of patients with active pulmonary sarcoidosis that were either followed without therapy or treated with corticosteroids. In those individuals who were untreated, on the average, the lung T-cell spontaneous release of IL-2 and proliferation continued and there was little change in lung function. In contrast, in those treated, the lung T-cell spontaneous release of IL-2 stopped, lung T-cells proliferation ceased, the numbers of lung helper T-cells were reduced, and there was marked improvement in lung volumes and diffusing capacity.

Interestingly, when evaluated in vitro, although cyclosporine efficiently suppresses the spontaneous expression of interleukin-2 gene and T cell proliferation of lung T-lymphocytes recovered from the lower respiratory tract of individuals with active pulmonary sarcoidosis, administration of cyclosporine to individuals with active pulmonary sarcoidosis did not have any effect on lung T-cell IL-2 release, spontaneous proliferation, or lung helper cell accumulation, or lung function. The reasons for this likely center on the inability of cyclosporine to gain access to the lung parenchyma in sufficient concentrations to suppress the activated T-helper lymphocyte processes in doses that can be safely administered.

The trigger for the activated helper T-cell processes in the lower respiratory tract in pulmonary sarcoid is not known. One possibility is that the T-cells are infected with a retrovirus that alters their response to antigens or makes them autonomous in terms of their normal control. However, evaluation of lung T-lymphocytes for the presence of reverse transcriptase were negative as was the evaluation of these cells for the presence of the HTLV-1 retrovirus, the virus responsible for some human T-cell lymphomas. An alternative hypothesis for the pathogenesis of sarcoidosis is that it is due to a variety of antigens that the immune system responds to in an undamped fashion. Consistent with this concept, antigen presentation by alveolar macrophages to autologous T-lymphocytes is markedly enhanced in sarcoidosis. It is also possible that sarcoidosis is an "auto-immune" disorder in which antibodies directed against the T-cell antigen receptor may stimulate T-helper cells to proliferate in an enhanced manner. Consistent with this concept, there are anti-T-cell antibodies in the blood and lung of individuals with sarcoidosis. Detailed evaluation of these antibodies demonstrated that there are mostly of the IgM variety and are directed both to LEU-2 (suppressor/cytotoxic) T-cells as well as LEU-3 (helper/inducer) T-cells. However, the function of these anti-T-cell IgM antibodies is not known and they do not appear to effect T-cell proliferation independently or in conjunction with normal mechanisms of antigen stimulation. An alternative hypothesis to the pathogenesis of sarcoidosis is to consider marked expansion of T-helper cells in the sarcoid lung resulting from defect in T-suppressor cell networks. For example, T-suppressor cells may not proliferate and become activated in response to normal stimuli. To evaluate this hypothesis, we evaluated T cells recovered by bronchoalveolar lavage from patients with sarcoidosis and normal individuals to determine the proportions of T-suppressor cells which were simultaneously expressing surface antigens associated with activation (4F2, HLA-DR) or the interleukin-2 receptor that allows interleukin-2 to signal T-cells to proliferate. These studies demonstrated that although suppressor cells in patients with sarcoid can be activated, they do not appear to be proliferating in the sarcoid lung even though they can respond to a proliferation signal and express the interleukin-2 receptor. These observations suggest that the imbalance of T-helper and T-suppressor cells in the sarcoid lung may be associated with a lack of the signals and/or interaction required for the T-suppressor population to proliferate, thus allowing the T-helper populations to expand without the concomitant influence of the T-suppressor cells.

Alpha 1-antitrypsin (AAT), a 52,000 dalton serum glycoprotein produced by hepatocytes and mononuclear phagocytes, functions to inhibit neutrophil elastase, a proteolytic enzyme capable of destroying all protein components of connective tissue. The AAT gene is highly pleomorphic; more than 30 different haplotypes have been described. The AAT phenotype, referred to as the Pi (protease inhibitor) type, represents the codominant expression of the two parental AAT haplotypes. The most common AAT haplotypes in the U.S.A. are those of the M-family (M1, M2, M3;

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The classic approach to detecting the S and Z genes is at the protein level through a combination of serum isoelectric focusing, serum AAT levels and family studies. Recent studies have demonstrated that, utilizing synthetic oligonucleotide probes 19 bases in length, under appropriate hybridization conditions, single base differences can be detected in the AAT gene in genomic DNA. With four oligonucleotide probes, two complimentary to the M and Z difference in exon V, respectively, and two complimentary to the M and S difference in exon III, respectively, accurate diagnosis of all combinations of the S, Z, and M genes are possible at the level of genomic DNA. Importantly, using 4 oligonucleotide probes, it is possible to distinguish the "at risk" SZ heterozygote and ZZ homozygote from the "not at risk" MS and MZ heterozygote state and MM homozygote state.

The molecular basis for AAT deficiency for the common Z and S mutations have been defined and both result from point mutations in the coding region of the AAT gene. In both cases the mutant gene is transcribed, the mRNA translated and a protein is produced that differs from the normal M protein by single amino acid substitution. The mutant Z and S proteins functions normally as anti-neutrophil elastase. However, for reasons likely related to the inability of the AAT producing cells to process and secrete the mutant proteins in the normal fashion, for individuals with phenotypes ZZ and ZS, the amounts of AAT released by the AAT producing cells is markedly reduced, resulting in "AAT" deficiency and thus a deficiency in the lower respiratory tract of protection against neutrophil elastase.

In contrast to ZZ and SZ states, in which mutant proteins are found in reduced amounts, the "null-null" state is a rare form of AAT deficiency in which no AAT can be found at all. Since AAT normally provides almost all of the anti-neutrophil elastase protection of the lower respiratory tract, the lungs of "null-null" individuals are essentially defenseless against the burden of neutrophils, and they invariably develop emphysema early in adulthood. Recent studies evaluated AAT genes and AAT producing cells in an individual with AAT deficiency demonstrated that the "null-null" AAT gene represents a class of AAT mutant different from the Z and S mutants in that the deficiency of AAT associated with "null" represents the inability of the AAT gene to direct the synthesis of a detectable mRNA transcript. Furthermore, at least for the two null alleles represented in the family evaluated, restriction endonuclease mapping demonstrated that there was no major deletion, addition, rearrangement in the coding region or promoter region of the null genes.

Analysis of the human AAT gene using the restriction endonuclease Taq I has revealed a common polymorphism which is not associated with alteration of the AAT protein phenotype as defined by net charge. This polymorphism results from the loss Taq I recognition site approximately 1.1 kb 3' from exon V of the AAT gene and pedigree analysis reveals that it is inherited

in a mendelian fashion consistent with the autosomal codominant inheritance pattern of the AAT gene. While this Taq I restriction site polymorphism was found in 11.2% of the 116 AAT M1 protein haplotypes evaluated, it was not observed in any of the 98 AAT Z protein haplotypes studied. I.e., the Taq I polymorphism was clearly in linkage disequilibrium between M1 and Z AAT haplotypes. Although the significance of this polymorphism to the expression of AAT gene is unknown, these observations suggest that the categorization of AAT haplotypes could be developed at the DNA level that may help in the understanding the role of AAT and disease.

Although the liver is the major site of AAT synthesis in the body, mononuclear phagocytes, including blood monocytes and alveolar macrophages, also express the AAT gene. Analysis of mononuclear phagocytes of individuals with normal (MM) AAT phenotype using the full length cDNA probe for AAT demonstrated that monocytes and alveolar macrophages spontaneously express a single AAT mRNA of 1.7 kb, identical to that of liver. Quantification of the mRNA levels of demonstrated that monocytes AAT mRNA levels were 200-fold less than liver and that alveolar macrophage AAT mRNA levels were 70-fold less than liver, suggesting that on a per cell basis, mononuclear phagocytes are less able to express stable AAT gene transcript compared to liver, but that in the process of maturation for monocytes, alveolar macrophages likely up-regulate AAT gene expression. Consistent with this observation, an in vitro model of monocyte to macrophage maturation demonstrated 10-fold augmentation of average AAT mRNA level per cell. Importantly, analysis of alveolar macrophages of individuals with PiZZ type AAT deficiency demonstrated that while AAT mRNA levels were similar to that of alveolar macrophages of normal PiMM individuals, the PiZZ macrophages synthesize and secreted 10-fold less AAT consistent with the knowledge that the consequence of the AAT Z gene abnormality are expressed in mononuclear phagocytes as well as in liver. Although the total mass AAT produced by mononuclear phagocytes is probably far less than the mass produced by the liver, the fact that alveolar macrophages reside directly at the site where AAT functions to inhibit neutrophil elastase suggests that alveolar macrophages may play a role in the AATneutrophil elastase balance in the lower respiratory tract in both normals and in AAT deficiency.

It is known that oxidants and tobacco smoke can inactivate AAT in vitro. Studies have shown that AAT from smokers lungs may also be partially inactivated, in part explaining the high incidence of emphysema associated with cigarette smoking. Oxidative inactivation of AAT is probably due to modification of the methionine residue (met^{358}) at the P1 subsite position of the elastase binding site of the protein. To study the possibility of modulating the biological properties of AAT, we introduced selected sequence modifications at the reactive site by in vitro mutation of a clone AAT cDNA. Recent studies demonstrated that one AAT analogue produced in E.coli (directed by a cDNA altered by oligonucleotide directed mutagenesis) (met^{358} to val^{358}) was fully active as an elastase inhibitor but was also resistent to oxidative inactivation. A second protein (AAT met^{358} to arg^{358}) no longer inhibited elastase but was an efficient thrombin inhibitor. The active site of this latter molecule is identical to that of AAT-Pittsburgh variant which was associated with a fatal bleeding disorder.

Another process that may contribute to the oxidation of AAT in cigarette smokers is through oxidants released by inflammatory cells in the lower respiratory tract. To evaluate this concept, we examined the ability of

alveolar macrophages recovered from the lungs of cigarette smokers to spontaneously oxidize AAT. Alveolar macrophages from smokers spontaneously release more superoxide and hydrogen peroxide than do alveolar macrophages from non-smokers. Consistent with the spontaneous release of oxidants by smokers' alveolar macrophages, AAT incubated in a chamber separated by dialysis membrane from smokers alveolar macrophages was clearly inactivated. In this context, alveolar macrophages of smokers may play a major role in the pathogenesis of emphysema by inactivating the major antielastase defense in the lower respiratory tract.

The emphysema associated with AAT deficiency results from insufficient amounts of AAT in the blood to protect the lung from destruction by neutrophil elastase. To evaluate the concept that intermittent parenteral therapy with AAT can re-establish normal lung antineutrophil elastase defenses, 15 AAT deficient individuals are being treated with 60 mg/kg purified human AAT once weekly. The evaluation by bronchoalveolar lavage before at 2 and 6 days after infusion demonstrated that lung epithelial lining fluid AAT levels are markedly increased and brought into the normal range. Most importantly, therapy with AAT resulted augmentation of the antineutrophil capacity of the epithelial lining fluid and there was good correlation between the epithelial lining fluid AAT levels and epithelial lining fluid elastase inhibitory capacity. These observations are consistent with the concepts that blood AAT levels dictate lung AAT levels, AAT epithelial lining fluid levels define lung antineutrophil elastase defenses, and weekly parenteral therapy was 60 mg/kg AAT can reestablish the lung anti-neutrophil elastase defenses of individuals deficient in AAT, providing a logical approach to the treatment of this disorder.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02407-11 PB

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Destructive Lung Disease

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ronald G. Crystal, M.D.	Chief	Pulmonary Branch, NHLBI
Others:	Mark Brantly	Senior Staff Fellow	Pulmonary Branch, NHLBI
	Anthony Casolaro	Senior Staff Fellow	Pulmonary Branch, NHLBI
	Robert Garver	Senior Staff Fellow	Pulmonary Branch, NHLBI
	Jean Francois Mornex	Guest Researcher	Pulmonary Branch, NHLBI
	Toshihiro Nukiwa	Guest Researcher	Pulmonary Branch, NHLBI

COOPERATING UNITS *(if any)*
Michael Courtney, Jean Pierre LeCoq - Transgene, Strasbourg, France

LAB/BRANCH
Pulmonary Branch

SECTION

INSTITUTE AND LOCATION
NHLBI:NIH: Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
12.7	8.7	4

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

There are 2 million individuals in the U.S.A. with emphysema. Two percent develop the disease because of inheritance of a deficiency of alpha 1-antitrypsin (AAT), an antiprotease that protects the lower respiratory tract from destruction mediated by elastase released by neutrophils. Oligionucleotides have been used to detect specific mutations in the AAT gene. The "null" AAT state is associated with an intact gene but no detectable AAT mRNA. Alveolar macrophages produce AAT, thus providing the protein at the site of disease. Site directed mutagenesis has been used to produce a recombinant AAT molecules in E.coli that is oxidation resistant. Therapy of AAT deficiency with AAT purified from pooled plasma has demonstrated that the anti-neutrophil-elastase defenses of the lung can be re-established with intermittent intravenous administration of 60 mg/kg AAT.

940

Others: (Cont.)

Fumitaka Ogushi	Guest Reseacher	Pulmonary Branch, NHLBI
Lester Paul	Senior Staff Fellow	Pulmonary Branch, NHLBI
Ken Satoh	Visiting Fellow	Pulmonary Branch, NHLBI
Sherwin Straus	Senior Staff Fellow	Pulmonary Branch, NHLBI
Mark Wewers	Senior Staff Fellow	Pulmonary Branch, NHLBI

Objectives

The disorders characterized by destruction of the alveolar walls are commonly called emphysema. Of the $2x10^6$ individuals in the USA with emphysema, approximately 98% acquire the disease, usually on the basis of cigarette smoking, while 2% have an inherited disorder called alpha-1-antitrypsin deficiency. All forms of emphysema are characterized by a dissolution of the lung parenchyma. The inflammation that causes these changes is dominated by alveolar macrophages together with smaller numbers of neutrophils.

Alpha 1-antitrypsin (AAT), a 52,000 dalton serum glycoprotein produced by hepatocytes and mononuclear phagocytes, functions to inhibit neutrophil elastase, a proteolytic enzyme capable of destroying all protein components of connective tissue. The AAT gene is highly pleomorphic; more than 30 different haplotypes have been described. The AAT phenotype, referred to as the Pi (protease inhibitor) type, represents the codominant expression of the two parental AAT haplotypes. The most common AAT haplotypes in the U.S.A. are those of the M-family (M1, M2, M3; combined frequency greater than 0.90), the S type (frequency 0.02-0.04) and the Z type (0.01-0.02). The clinical interest in these AAT haplotypes is based on the knowledge that inheritance of the phenotypes PiZZ and PiSZ is associated with an increased risk for the development of emphysema (in adults) and/or liver disease (in children). Studies at the gene and protein levels have shown that the S and Z genes each code for proteins that differ from the M protein by a single amino acid; in the S protein val^{264} replaces glu^{264} on the Z protein lys^{342} replaces glu^{342}.

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Significance to Biomedical Research

There are approximately 2 million individuals in the United States with emphysema. Using alpha 1-antitrypsin deficiency as the "model" of emphysema, insights have been gained as to pathogenesis of the destructive process in the lower respiratory tract. For alpha 1-antitrypsin deficiency which represents approximately 2% of these individuals, replacement therapy with alpha 1-antitrypsin purified from pooled plasma is a reality. Within the next few years, it should be possible to evaluate the use of alpha 1-antitrypsin produced by recombinant DNA technology. With this background, it should be possible to develop an understanding of the pathogenesis and strategy for therapy to the acquired form of emphysema.

Proposed Course

Studies will be continung to evaluate the pathogenesis of both hereditary and acquired forms of emphysema and to test therapies to suppress those processes that cause destruction of the lower respiratory tract.

Publications

Gadek JE, Fells GA, Zimmerman RL, Crystal RG. Role of connective tissue proteases in the pathogenesis of chronic inflammatory lung disease. Environmental Health Perspectives 1984; 55:297-306.

Fukida Y, Ferrans VJ, Crystal RG. Development of elastic fibers of nuchal ligament, aorta and lung of fetal and postnatal sheep. Am J Anat 1984; 170:597-629.

Mornex JF, Crystal RG. Protease-antiprotease imbalance in lung disease. In: Arnaud P, Bienvenu J, Laurent P, eds., Markers Proteins in Inflammation, Vol 2. New York and Berlin: Walter de Gruyter 1984; 261-270.

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Davidson JM, Shibahara S, Schafer MP, Harrison M, Leach C, Tolstoshev P. Crystal RG. Sheep elastin genes: isolation and preliminary characterization of a 9.9 kilobase genomic clone. Biochem J 1984; 220:643-652.

Courtney M, Jallat A, Tessier LH, Benavente A, Crystal RG, LeCoq JP. Synthesis in E.coli of alpha 1-antitrypsin variants with potential in the therapy of emphysema and thrombosis. Nature 1985; 313:149-151.

Straus SD, Fells GA, Wewers MD, Courtney M, Tessier L-H, Tolstoshev P, LeCoq J-P, Crystal RG. Evaluation of recombinant DNA-directed E.coli produced α1-antitrypsin as an anti-neurophil elastase for potential use as replacement therapy of α1-antitrypsin deficiency. Biochem Biophys Res Comm 1985;130:1177-1184.

Publications (Cont.)

Pelham F, Wewers M, Crystal RG, Buist SA, Janoff A. Urinzary excretion of desmosine (elastin crosslinks) in subjects with PiZZ alphal-antitrypsin deficiency, a phenotype associated with hereditary predisposition to pulmonary emphysema. Am Rev Resp Dis (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02533-01 PB

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms of Fibrosis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ronald G. Crystal, M.D., Chief		Pulmonary Branch, NHLBI
Others:	Peter Bitterman	Medical Staff Fellow	Pulmonary Branch, NHLBI
	Paul Basset	Guest Worker	Pulmonary Branch, NHLBI
	Andre Cantin	Guest Worker	Pulmonary Branch, NHLBI
	Takeo Hirata	Guest Worker	Pulmonary Branch, NHLBI
	Richard Hubbard	Senior Staff Fellow	Pulmonary Branch, NHLBI

COOPERATING UNITS *(if any)*
Pathology Branch, ODIR, NHLBI, NIH, Victor Ferrans; Hospital Bichart, INSERM, Paris, Francois Basset; Laboratory Clinical Investigation, NIAID, Eric Ottenson; Laboratory of Developmental Biology and Anomalies, NIDR, George Martin.

LAB/BRANCH
Pulmonary Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
11	7	4

CHECK APPROPRIATE BOX(ES)

- [X] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [X] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The fibrotic lung disorders represent 15% of the non-infectious, non-malignant lung diseases; they are often progressive and can be fatal. The fibrosis results from damage caused by inflammatory cells and subsequent proliferation of mesenchymal cells, driven by mediators released by alveolar macrophages. The primary mediators are fibronectin and alveolar macrophages derived growth factor. Other mediators include interleukin-1 and a platlet derived growth factor-like protein, likely coded by the c-sis proto-oncogene. With knowledge of the specific processes involved, strategies can be developed to modulate these mediators as therapy for these disorders.

GPO 914-918

Others Investigators (Cont.)

William Rom	Guest Workers	Pulmonary Branch, NHLBI
James Willey	Senior Staff Fellow	Pulmonary Branch, NHLBI
Kohei Yamauchi	Visiting Associate	Pulmonary Branch, NHLBI

Objectives

The disorders characterized by progressive fibrosis of the lung parenchyma represent a large subgroup of the interstitial lung disorders, chronic disorders in which derangements of the alveolar wall are accompanied by "fibrosis", an accumulation of mesenchymal cells and the collagenous matrix produced by these cells. In general, the inflammation of these disorders is dominated by alveolar macrophages, and to a lesser extent, neutrophils and/or eosinophils. Example of these fibrotic disorders include idiopathic pulmonary fibrosis and asbestosis.

The current concepts of the mechanisms of pulmonary fibrosis hold that the accumulation of fibroblast and fibroblast products result from two general processes. First, there must be damage to the alveolar wall; in most cases this is mediated by products of inflammatory cells, primarily neutrophils and/or eosinophils but including macrophages. Second, that there is enhanced proliferation of mesenchymal cells in the alveolar walls driven by growth signals released by alveolar macrophages.

The bulk of the injury caused by neutrophils, eosinophils and macrophages result from the ability of these cells to release toxic oxidant radicals such as superoxide, hydrogen peroxide and hydroxyl radical. In addition, neutrophils, and to a lesser extent eosinophils and young macrophages, can release peroxidases that, in the presence of hydroxyl radical and a halide such as the chloride anion, produce the toxic hypohalide radical. Recent studies have demonstrated that the lung has protection on its epithelial surface to prevent oxidant mediated injury. Included among these antioxidants is glutathione, a tripeptide that is an effective inhibitor of hydrogen peroxide. The glutathione levels on the epithelial surface of the human lower respiratory tract are seventy times that in plasma. Interestingly, the glutathione levels in the epithelial lining fluid of the lungs of cigarette smokers is higher than that of normal while the glutathione levels in patients with idiopathic pulmonary fibrosis are lower. While the mechanisms of the accumulation of glutathione in the lower respiratory tract are not known, it is clear that alveolar macrophages release glutathione as do some parenchymal cells. In addition to glutathione, there are other small molecular weight antioxidants in the epithelial lining fluid including vitamin E. Furthermore, there are large molecular weight protein antioxidants including catalase. Although catalase is an intracellular antioxidant, it is found in large amounts on the epithelial surface of the lower respiratory tract, presumably released by epithelial cells during their process of normal cell turnover. Independent of the mechanisms for the accumulation of catalase, it is a very effective antioxidant against hydrogen peroxide and thus affords significant protection to the lower respiratory tract.

Although eosinophils are classically considered to be a "protective" cell for inflammatory and immune processes, there is increasing evidence that the eosinophil can effect damage to normal tissues. To evaluate this concept in the lower respiratory tract in man, it is necessary to find a "pure" eosinophilic inflammatory process. To accomplish this, in conjunction with the Laboratory of Clinical Investigation of the National Institute of Allergy and Infectious Diseases and the Tuberculosis Research Center, Madras, India, and the Indian Council for Medical Research, under the auspices of the Indo-USA joint agreement on technology, members of the Pulmonary Branch evaluated patients with acute tropical pulmonary

eosinophilia in Madras, India. These individuals have a pure eosinophil inflammatory process in the lower respiratory tract that includes up to 70% of the total inflammatory cells being eosinophils (normal less than 1%). When treated with diethylcarbamezine, the eosinophil inflammatory process is suppressed and the patients improve. The preliminary study was followed up with the study of patients that had been previously treated with diethylcarbamezine and were reevaluated six months to two years later. Strikingly, they continued to have a low level of an eosinophil inflammatory process in the lower tract with concomitant evidence of a mild fibrotic lung disease. Evaluation of the inflammatory cells recovered by bronchoalveolar lavage revealed that they were releasing increased amounts of superoxide and hydrogen peroxide (compared to normal inflammatory cells). Furthermore, when treated with corticosteroids, the level of oxidant production by these cells diminished.

The inorganic dust disorders including asbestosis, silicosis, and coal worker pneumoconiosis are all characterized by damage to the parenchymal cells of the lower respiratory tract. Evaluation of the inflammatory cells of nonsmoking individuals exposed for long periods to these inorganic dusts demonstrates that, in many cases, the alveolar macrophages are releasing superoxide and hydrogen peroxide. In this context, one way to approach the therapy of these disorders is to augment the antioxidant screen of the lower respiratory tract or to suppress the production of these oxidants by the inflammatory cells that have accumulated.

All of the fibrotic lung diseases are characterized by an increase in the number of alveolar macrophages in the lower respiratory tract. The classic concept of accumulation of mononuclear phagocytes in tissues suggests that there is an influx of monocytes to the tissues likely called in by chemotactic factors. In individuals exposed to asbestos, evaluation of alveolar macrophages recovered by bronchoalveolar lavage revealed that a significant number of the macrophages on the epithelial surface of the lung express monocyte lineage antigens, consistent with the concept that they represented newly recruited monocytes. Furthermore, evaluation of alveolar macrophages from individuals with a variety of fibrotic lung diseases, including asbestos exposure, has demonstrated that a proportion of these macrophages are proliferating, suggesting that enhanced replication in situ also contributes to the process of macrophage accumulation. In this context, it is known that for all mammalian cells to proliferate they require iron. The major, if not the only, mechanism by which iron gains access to mammalian cells is through the carrier protein transferrin. Interestingly, while blood monocytes, the precursor of the alveolar macrophage, do not have transferrin receptors, alveolar macrophages do. Characterization of human alveolar macrophage transferrin receptors by monoclonal antibodies, receptor-ligand binding studies, and evaluation of transferrin receptor mRNA levels has confirmed this concept. Thus, although the mediators that stimulate alveolar macrophage to proliferate in these diseases are not known, the alveolar macrophages do have at least one receptor which is likely necessary to allow it to proliferate.

Tissue fibrosis results, in part, from an interaction between growth regulatory molecules released by mononuclear phagocytes and the fibroblast target cells. In the chronic interstitial lung disorders, alveolar macrophages are known to be spontaneously releasing increased amounts of two growth factors for fibroblasts, fibronectin and alveolar macrophage derived growth factor (AMDGF), that together stimulate nonreplicating lung fibroblasts to divide. In addition to these two

primary growth signals, alveolar macrophages also release other mediators that may have the potential role in modulating lung fibroblast replication. However, recent evaluation of several of these mediators, including interferon-γ and interleukin-1, suggests they have little (interleukin-1) or no effect (interferon-γ). Prostaglandin E_2, another macrophage product, suppresses fibroblast replication in a dose-dependent manner.

Recent studies have shed additional light on the genes involved in growth factor production by mononuclear phagocytes. These studies have shown that activated human blood monocytes release a mediator that is capable of attracting smooth muscle cells and cooperating with other mediators to stimulate fibroblasts to divide. Purification of this mediator demonstrated that its chromatographic properties and chemical stability had a close similarity to platelet derived growth factor (PDGF). Consistent with this concept, the partially purified monocyte mediator displaced ^{125}I-labelled PDGF from its receptor on BALB/c-3T3 cells and immunoprecipitated with antibodies directed against PGDF. Strikingly, conditions which stimulated monocytes to release this PGDF-like molecule also stimulated the monocytes to express the gene coding for the c-sis proto-oncogene, a gene on chromosome 22 known to code for one of the PDGF chains. In contrast, resting monocytes did not secrete the PDGF-like molecule nor express detectable amounts of c-sis mRNA. These observations are consistent with the concept that expression of the c-sis proto-oncogene may play a role in enabling mononuclear phagocytes to modulate the local accumulation of mesenchymal elements and have implications for the process of normal wound healing as well as for the pathogenesis of several common chronic human disorders. In the lung, studies have demonstrated that alveolar macrophages express the c-sis proto-oncogene and are capable, when stimulated, of releasing a PDGF-like molecule. Furthermore, in idiopathic pulmonary fibrosis, alveolar macrophages are releasing markedly increased amounts of these PDGF-like molecules compared to normal resting alveolar macrophages. Thus, while the role of this PDGF-like molecule in the pathogenesis of the fibrotic lung diseases is not clear, it does appear that expression of the c-sis proto-oncogene correlates with the release of a functional molecule by these cells.

To gain insight into the early mechanisms of fibrosis in idiopathic pulmonary fibrosis, three families with familial pulmonary fibrosis have been evaluated in detail. The disease familial pulmonary fibrosis shares with idiopathic pulmonary fibrosis a characteristic pattern of alveolar inflammation featuring neutrophil accumulation and macrophage activation. Strikingly, despite being clinically normal in every respect, about half of the first degree relatives of individuals with familial pulmonary fibrosis have clear evidence of a similar pattern of alveolar inflammation despite the fact that they have no evidence of structural damage to their lower respiratory tract. This observation raises the intriguing issue of whether these individuals are at risk of developing disease in the future.

In collaboration with INSERM, Paris, and the Pathology Branch, NHLBI, a recent study of several hundred biopsies of patients with interstitial lung disease has demonstrated that the classic view of the "interstitial" nature of the fibrosis in these diseases is too simplified. It is apparent from the ultrastructural evaluation of these biopsies that intraalveolar fibrosis also plays an important role and that incorporation of this intraalveolar process into the wall of the

alveoli contributes to the expansion of the thickness of the alveolar wall with fibroblast and fibroblast products.

In the context of the mechanisms of pulmonary fibrosis discussed above, it is apparent that therapy should be directed to suppressing the inflammatory cells that cause the damage to the alveolar walls and to the inflammatory cells (i.e., macrophages) that are driving the fibroblasts to proliferate. In this regard, a study was carried out comparing corticosteroids to cyclophosphamide for their ability to suppress the neutrophil component of the alveolitis in idiopathic pulmonary fibrosis. On the average, glucocorticoids had little effect while cyclophosphamide markedly suppressed the numbers of neutrophils in the lung when cyclophosphamide was used alone or in combination with corticosteroids. Like their effect on the neutrophil component of the inflammation, glucocorticoids have little effect on the release of fibronectin and AMDGF by alveolar macrophages of patients with fibrotic lung disease. This is despite the fact that alvoelar macrophages have glucocorticoid receptors on their surface. Interestingly, colchicine in doses that likely can be achieved in vivo will suppress the release of fibronectin and AMDGF, at least in part, by alveolar macrophages of these patients.

Significance to Biomedical Research and the Program of the Institute

The fibrotic lung disorders are often progressive and can be fatal. Over all, they represent approximataely 15% of the non-infectious, non-malignant lung disorders in the United States. By understanding the processes by which the fibrosis occurs, it should be possible to design rational strategies to stage and treat these disorders.

Proposed Course

Work over the next several years will involve isolation of the specific genes and their products involved in driving the accumulation of fibroblasts in the lung parenchyma. As each of these mechanisms become apparent, strategies will be developed to attempt to modulate these genes in clinical studies.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HL 02534-01 PB

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
T-Lymphocyte Disorders

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ronald G. Crystal, M.D., Chief		Pulmonary Branch, NHLBI
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	Theodore McLemore	Senior Staff Fellow	Pulmonary Branch, NHLBI
	Mark Liu	Senior Staff Fellow	Pulmonary Branch, NHLBI
	Yves Martinet	Guest Worker	Pulmonary Branch, NHLBI

COOPERATING UNITS *(if any)*
none

LAB/BRANCH
Pulmonary Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10.5	6.5	4

CHECK APPROPRIATE BOX(ES)
☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The T-lymphocyte lung disorders occur in 20 to 50 per 100,000 of the U.S.A. population. The "model" disorder of this group is sarcoidosis, a disease characterized by the accumulation of activated helper T-lymphocytes at the sites of disease. These T-cells spontaneously express the interleukin-2 gene, thus driving T-cells in the local milieu to proliferate. Treatment of these individuals with corticosteroids results in suppression of interleukin-2 gene expression, cessation of lung T-cell proliferation, and improvement in lung function.

Other Investigators:

Paula Pinkston	Medical Staff Fellow	Pulmonary Branch, NHLBI
Cesare Saltini	Visiting Scientist	Pulmonary Branch, NHLBI
John Spurzem	Guest Worker	Pulmonary Branch, NHLBI

Objectives

The disorders characterized by the accumulation of T-lymphocytes in the lower respiratory tract are also a subgroup of the interstitial lung disorders. However, although occasionally associated with progressive fibrosis of the lung parenchyma late in their course, these disorders are universally characterized by large numbers of T-lymphocytes that dominate the inflammation and cause dysfunction by their presence, which distorts the alveolar, bronchial, and vascular walls, thus modifying the intimate relationship between air and blood. Examples of the T-lymphocytes disorders include sarcoidosis, berylliosis and hypersensitivity pneumonitis.

Pulmonary saracoidosis is a disorder of the lower respiratory tract characterized by chronic inflammation, granuloma formation and, in some individuals, parenchymal fibrosis. Together these processes derange the alveoli, airways and blood vessels, consequently impairing the ability of the lung to exchange gas in the normal fashion. As with the other interstitial lung disorders, it is recognized that the inflammation proceeds the other abnormalities that characterize this disorder. The inflammation of active pulmonary sarcoid is dominated by an accumulation of T-helper lymphocytes in the lung parenchyma. These T-cells are thought to play a central role in the pathogenesis of sarcoidosis in two ways. First, the accumulated T-cells distort the architecture of the parenchyma, thus altering the intimate relationships between air and blood. Second, the T-cell populations are activated and spontaneously releasing monocyte chemotactic factor and interferon gamma, mediators that recruit and activate mononuclear phagocytes, respectively, events that are early steps in the process of granuloma formation. In this context, an understanding of the pathogenesis of pulmonary sarcoidosis is intimately linked to the understanding of the process directing the accumulation of T-lymphocytes in the lower respiratory tract of individuals with active disease. Relevant to this question, prior work in this laboratory has demonstrated that the T-lymphocytes recovered from the lungs of these patients are spontaneously proliferating and spontaneously releasing interleukin-2 (IL-2), the T-cell growth factor. Thus, while the stimulus that initiates the process is unknown, the IL-2 releasing lung T-cells are thought to be responsible for maintaining the T-cell inflammation and thus maintaining the disease in an active state.

Recent studies have expanded upon this concept by determining that the IL-2 releasing cells in the lung are LEU3+ (helper-inducer) T-lymphocytes expressing HLA-DR antigens on their surface. Since this is the same subset of T-lymphocytes that likely release IL-2 in normal antigen driven T-cell proliferation, these observations lend credence to the concept that the T-cell inflammation of sarcoid is an exaggeration of the normal processes of T-cell activation. Consistent with this concept, studies utilizing an IL-2 cDNA probe have demonstrated that lung T-lymphocytes from individuals with active pulmonary sarcoidosis are spontaneously expressing the IL-2 gene while blood T-cells from the same individuals are not.

Since the IL-2 driven accumulation of helper T-lymphocytes in the lung plays a central role in the pathogenesis of sarcoidosis, it is reasonable to hypothesize if the interleukin-2 gene expression could be suppressed in lung T-cells, these cells would be prevented from accumulating in the lung parenchyma. To test this hypothesis, we prospectively evaluated the lung T-cells and lung function in a group of patients with active pulmonary sarcoidosis that were either followed

without therapy or treated with corticosteroids. In those individuals who were untreated, on the average, the lung T-cell spontaneous release of IL-2 and proliferation continued and there was little change in lung function. In contrast, in those treated, the lung T-cell spontaneous release of IL-2 stopped, lung T-cells proliferation ceased, the numbers of lung helper T-cells were reduced, and there was marked improvement in lung volumes and diffusing capacity.

Interestingly, when evaluated in vitro, although cyclosporine efficiently suppresses the spontaneous expression of interleukin-2 gene and T cell proliferation of lung T-lymphocytes recovered from the lower respiratory tract of individuals with active pulmonary sarcoidosis, administration of cyclosporine to individuals with active pulmonary sarcoidosis did not have any effect on lung T-cell IL-2 release, spontaneous proliferation, or lung helper cell accumulation, or lung function. The reasons for this likely center on the inability of cyclosporine to gain access to the lung parenchyma in sufficient concentrations to suppress the activated T-helper lymphocyte processes in doses that can be safely administered.

The trigger for the activated helper T-cell processes in the lower respiratory tract in pulmonary sarcoid is not known. One possibility is that the T-cells are infected with a retrovirus that alters their response to antigens or makes them autonomous in terms of their normal control. However, evaluation of lung T-lymphocytes for the presence of reverse transcriptase were negative as was the evaluation of these cells for the presence of the HTLV-1 retrovirus, the virus responsible for some human T-cell lymphomas. An alternative hypothesis for the pathogenesis of sarcoidosis is that it is due to a variety of antigens that the immune system responds to in an undamped fashion. Consistent with this concept, antigen presentation by alveolar macrophages to autologous T-lymphocytes is markedly enhanced in sarcoidosis. It is also possible that sarcoidosis is an "auto-immune" disorder in which antibodies directed against the T-cell antigen receptor may stimulate T-helper cells to proliferate in an enhanced manner. Consistent with this concept, there are anti-T-cell antibodies in the blood and lung of individuals with sarcoidosis. Detailed evaluation of these antibodies demonstrated that there are mostly of the IgM variety and are directed both to LEU-2 (suppressor/cytotoxic) T-cells as well as LEU-3 (helper/inducer) T-cells. However, the function of these anti-T-cell IgM antibodies is not known and they do not appear to effect T-cell proliferation independently or in conjunction with normal mechanisms of antigen stimulation. An alternative hypothesis to the pathogenesis of sarcoidosis is to consider marked expansion of T-helper cells in the sarcoid lung resulting from defect in T-suppressor cell networks. For example, T-suppressor cells may not proliferate and become activated in response to normal stimuli. To evaluate this hypothesis, we evaluated T cells recovered by bronchoalveolar lavage from patients with sarcoidosis and normal individuals to determine the proportions of T-suppressor cells which were simultaneously expressing surface antigens associated with activation (4F2, HLA-DR) or the interleukin-2 receptor that allows interleukin-2 to signal T-cells to proliferate. These studies demonstrated that although suppressor cells in patients with sarcoid can be activated, they do not appear to be proliferating in the sarcoid lung even though they can respond to a proliferation signal and express the interleukin-2 receptor. These observations suggest that the imbalance of T-helper and T-suppressor cells in the sarcoid lung may be associated with a lack of the signals and/or interaction required for the T-suppressor population to proliferate, thus allowing the T-helper populations to expand without the concomitant influence of the T-suppressor cells.

Significance to Biomedical Research and the Program of the Institute

The T-lymphocyte disorders of the lower respiratory tract have a prevalence of 20 to 50 per 100,000 in the U.S.A. population. Approximately, 50% of these patients have permanent dysfunction of the lung and 10% eventually die from the disorder. The mechanisms of how these T-lymphocytes accumulate in the lung are beginning to be understood. With this understanding, it should be possible to design rational strategies to stage and treat these individuals.

Proposed Course

Studies will continue to decipher the mechanisms by which T-lymphocytes accumulate in the lower respiratory tract in each of these disorders and to understand the function of the individual T-lymphocyte subsets in the pathogenesis of the disease states. As these mechanisms are understood, strategies will be developed to attempt to modulate these processes in clinical trials.

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