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INTRAMURAL RESEARCH

THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

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THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
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ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
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N18TG-2 mouse neuroblastoma cells were fused with 18-day rat embryo retina cells, and somatic cell hybrid lines then were established to obtain clonal cell lines that continue to express genes characteristic of cells in retina. One of the hybrid cell lines generated, N18RE-103 possesses high tyrosine hydroxylase activity and synthesizes dopamine; whereas, tyrosine hydroxylase is not expressed by the parental N18TG-2 cells. N18RE-103 cells also possess voltage-sensitive $Na^+$, $K^+$, and $Ca^{2+}$ channels. N18RE-103 cells were used as an immunogen for the production of hybridoma cell lines that synthesize monoclonal antibodies to antigens expressed by N18RE-103 cells and retina. Thirty-three of the 374 hybridoma cell lines obtained synthesize antibodies to antigens expressed by N18RE-103 cells and rat retina cells, but not with cell lines from other tissues. Some of the antigens recognized by the monoclonal antibodies are expressed by different cell types in adult rat retina. For example, antibody 41C5 binds to antigen expressed by some, but not all, retinal ganglion neurons. Antibody 41A4 binds to antigen associated with the ganglion neuron layer. Antibody 89A5 binds to molecules restricted predominantly to the outer segments of photoreceptor cells. Antibody 38B6 binds to an abundant antigen on photoreceptor cell bodies and to less abundant antigen associated with horizontal, bipolar, and amacrine neuron soma. In contrast, antibody 35F8 recognizes antigen distributed in a punctate manner in the inner nuclear layer, the inner synaptic layer, and ganglion neuron layer of retina.

A λgt11 cDNA expression library was prepared and the recombinants were screened with antibody 41C5. One positive recombinant clone, λ41C5, was detected, which directs the synthesis of a 41C5-β galactosidase fusion protein recognized by antibody 41C5. Northern blot analysis with N18RE-103 poly $A^+$ RNA revealed one species of 41C5 poly $A^+$ RNA with a chain length of approximately 1 Kb. The nucleotide sequence of the λ41C5 insert was determined. One open reading frame was found, but no homology was detected between the predicted amino acid sequence of 41C5 and other proteins.

The distribution of 41C5 protein was shown by indirect immunofluorescence to be highly restricted to certain cells in rat retina and brain; the antigen was not detected in striated muscle, cardiac muscle, liver, or kidney. The distribution of 41C5 mRNA also was shown by <u>in situ</u> hybridization with a synthetic labeled

oligodeoxynucleotide probe to be highly restricted in rat retina and brain. 41C5 cDNA will be used as a probe to study the mechanisms that enable only a few cell types in the nervous system to express the 41C5 gene.

Hybridoma cell lines were generated that synthesize antibodies directed against cells from rat or rabbit retina that were used as immunogens. One monoclonal antibody detects an antigen that is most abundant in the outer layer of axons in the optic nerve. An antibody specific for the inner and outer synaptic layers of the retina was found as well as antibodies that bind only to Muller cells. Still other antibodies are specific for astrocytes in the retinal ganglion neuron layer. Some of the antigens that are markers of cell types were found to be proteins and were partially purified.

As described previously, 4 species of $\alpha_s$ cDNA clones were found that differ in nucleotide sequence in the region that corresponds to amino acid residues 71-88 in $\alpha_s$ protein, a subunit of $\alpha_s$ signal transduction protein. A mechanism was proposed for generating 4 species of $\alpha_s$ mRNA from a common precursor RNA transcribed from a single gene by 2 types of alternative splicing. Further work on $\alpha_s$ mRNA revealed that half the normal amount of $\alpha_s$ mRNA is present in fibroblasts of pseudo- hypoparathyroid patients. Levels of $\alpha_s$ mRNAs that code for low and high molecular weight forms of $\alpha_s$ protein were reduced with no apparent change in the ratio of the different species of $\alpha_s$ mRNA to one another. These results suggest that the genetic lesion in pseudohypoparathyroidism type Ia either decreases the rate of synthesis of a common precursor of the 4 species of $\alpha_s$ mRNA or increases equally the rates of turnover of the four species of $\alpha_s$ mRNA.

Two $\alpha_i$-lcDNA clones also were obtained from a human brain cDNA library that correspond to $\alpha_i$ protein subunits of $G_i$ signal-transduction proteins. One of the cDNA inserts was sequenced completely, the other partially. The nucleotide sequence of human brain $\alpha_i$-1 is similar to that of bovine brain $\alpha_i$-1, but differs significantly from $\alpha_i$-2 cDNAs from human monocytes, rat glioma cells, and mouse macrophages in nucleotide and amino acid sequences (88% and approximately 73% homology, respectively). The 3'-untranslated region of human brain $\alpha_i$-1 also differs markedly from that of human monocyte $\alpha_i$-2.

Five genomic DNA clones for $\alpha$ genes were obtained. One clone was identified as a gene for $\alpha_s$, and 2 clones, 1 from a human genomic DNA library, the other from a rat genomic DNA library, were identified as $\alpha_o$ genes. Further work is needed to identify the 2 remaining $\alpha$ genomic DNA clones. The focus in current studies is to define the nucleotide sequences of the 5'-upstream

regulatory regions of α genes.

A λgt11 cDNA library was constructed from poly $A^+$ RNA from 14 day chick embryo retina and was screened for recombinants with antibodies of β-subunits. One recombinant was detected with 9 out of 10 monospecific rabbit antibody preparations directed against the β-subunit of bovine transducin. The nucleotide sequence of the cDNA insert was determined and the predicted amino acid sequence was compared to that of the bovine β-subunit. Only a small region of homology was observed; hence, the cloned DNA is not related to the β-subunit. Six of 10 amino acid residues predicted for the recombinant DNA were identical to amino acid residues near the N-terminus of the β- subunit and 2 glutamine residues of the β-subunit were replaced by glutamic acid residues. A synthetic decapeptide corresponding to the amino-terminal residues of β blocked the binding of anti β antibodies to β-subunits. These results show that the amino terminal decapeptide of the β- subunit is a major antigenic site of the native protein.

L-Type voltage-sensitive calcium channels are sensitive to dihydropyridine agonists and antagonists. Activation of the L-type voltage-sensitive calcium channels of parathyroid cells opens the calcium channels and permits the entry of extracellular calcium ions into the parathyroid cells, which paradoxically, inhibits parathyroid hormone release. Calcium channel antagonists that block calcium entry into cells, stimulate secretion of the parathyroid hormone. Polyclonal, monospecific mouse antibodies to highly purified preparations of the α-subunit of voltage-sensitive calcium channels from rat T-tubules were obtained and were tested for their effects on parathyroid hormone secretion by cells. Three mouse antibody preparations blocked the secretion of parathyroid hormone from cells. Parathyroid cell protein was solublized and fractionated by SDS gel electrophoresis. The antibodies bound to only one major band of protein with an apparent $M_r$ of 150,000. Incubation of a mouse antibody with parathyroid cells resulted in the activation of voltage-sensitive calcium channels and markedly increased $Ca^{2+}$ uptake by parathyroid cells and inhibited parathyroid hormone release. These results suggest that the antibodies bind to α subunits of voltage-sensitive calcium channels of parathyroid cells and activate the calcium channels.

Previously we showed that elevation of cAMP levels of NG108-15 neuroblastoma-glioma hybrid cells or neuroblastoma cells for several days results in marked increases in the activities of voltage-sensitive $Na^+$, $K^+$, and $Ca^{2+}$ channels, and the rate of spontaneous secretion of acetylcholine at synapses between the hybrid cells and cultured striated muscle cells. We obtained cDNA clones for species of poly $A^+$ RNA that increase in abundance when

NG108-15 or NS20-Y cells are treated for 5 days with dibutyryl cAMP, as well as cDNA clones for other species of mRNA that decrease in abundance. The dibutyryl cAMP-dependent increases in poly $A^+$ RNA range from 5- to 90-fold above those of control cells, depending upon the species of mRNA examined. The levels of most species of poly $A^+$ RNA were not affected by treatment of cells with dibutyryl cAMP. Northern blot analysis showed that some independently isolated recombinant clones hybridize to the same species of mRNA; however, cDNA clones were obtained for approximately 15 species for dibutyryl cAMP responsive species of mRNA. A mouse genomic DNA library in Charon 30 was screened with cDNA probes for some dibutyryl cAMP responsive species of mRNA. Fragments of the cloned genomic DNA will be tested for promoter and/or enhancer activities.

TOP is a cell surface protein that is distributed topographically in a 35 fold gradient from the dorsal margin of chicken retina, which contains the highest concentration of TOP, to the ventral margin of the retina, which contains little TOP. An inverted gradient of TOP was detected in the tectum of 3 to 5 day chick embryos. The highest concentration of TOP is present in ventral tectum and the lowest is in dorsal tectum. The topographic map of cell position in the avian retina is inverted in its projection to the optic tectum. Dorsal retinal ganglion neuron axons project to ventral tectum and ventral retinal ganglion neurons axons project to dorsal tectum. Gradients of TOP are present in chick embryo retina and tectum before retinal axons arrive in the tectum. After 10 days of embryonic development, the number of antigenic TOP sites in the tectum decreases markedly; whereas, TOP levels in the retina remain relatively constant. The presence of corresponding TOP gradients in retina and tectum at the time of innervation of the tectum by retinal ganglion neuron axons suggests a possible role for TOP in orienting the dorsal-ventral axis of the retinal projection onto the tectum by homophilic interactions between TOP molecules.

The half-life of TOP in cultured retinal cells in the presence of cycloheximide or actinomycin D was 5 or 6 hr, respectively. Ablation of cells at the poles of the gradient in 60 hr chick embryos altered TOP expression during subsequent retinal development. Cells at the dorsal pole of the 13-day embryo retinas, 11.5 days after dorsal ablation, expressed 50% less TOP than normal and those at the ventral pole, after ventral ablation, expressed 300% more TOP than normal. Cells in other regions of the retina expressed normal levels of TOP.

Four neuropeptide Y (NPY) cDNA clones were found in two rat brain λgt11 cDNA libraries. These clones were sequenced and were found to contain 511 bp of rat prepro-NPY cDNA. The mRNA sequence revealed an open reading frame coding for a 98-amino acid gene

product containing a signal peptide, followed by a 36-residue sequence identical to human NPY, followed by a proteolysis/amidation signal Gly-Lys-Arg, followed by a 30-residue COOH-terminal peptide sequence that differs from the human counterpart in only two positions. This high degree of peptide sequence conservation is consistent with important physiological activities of both NPY and COOH-terminal peptides, an interesting finding since no function or activity is currently known for the COOH-terminal peptide.

The abundances of NPY mRNA (700-800 bases in length) in rat brain regions were determined by Northern and dot-blot analyses. The values (pg NPY mRNA/ug total RNA) are as follows: striatum, 6.1; frontal cortex, 5.0; hippocampus, 2.1; hypothalamus, 2.0; spinal cord, 1.8; medulla, 1.1; midbrain, 0.6; and cerebellum, 0.4. NPY mRNA also is abundant in adrenal gland, spleen, and heart and was detected in lung, skeletal muscle, stomach, and thyroid. The levels of immunoreactive NPY peptide were found by Drs. Higuchi and Yang to be parallel to NPY mRNA levels except for hypothalamus, which was richest in NPY peptide, and spleen, which has a low abundance of NPY peptide compared to its relatively high NPY mRNA level. The large amount of NPY mRNA in spleen suggests pro-NPY synthesis by immune cells; this possibility is being tested.

RNA from several well-studied rodent lines known to contain NPY were found to contain diverse levels of NPY mRNA. PC12 rat pheochromocytoma cells and N18TG-2 mouse neuroblastoma cells possess relatively low basal levels of NPY mRNA (0.25 and 0.13 pg/µg total RNA), while NG108-15 mouse neuroblastoma x rat glioma hybrid cells contain remarkably high amounts of NPY mRNA (11 pg/µg RNA). Treatment of PC12 cells with dexamethasone (Dex) for 24-96 hr increased the NPY mRNA abundance 2-5-fold. Elevation of the cAMP level with forskolin, or treatment with 8-bromo-cAMP elicited a rapid but transient increase in the NPY mRNA level in PC12 cells, forskolin alone elicited only slight increases (50%) in NPY mRNA, but the glucocorticoids increased NPY mRNA, but cAMP elevation markedly and persistently reduced the high basal NPY mRNA abundance by 85%. Cyclic GMP and non-glucocorticoid steroids had no effect.

Treatment of PC12 cells with phorbol 12-myristate 13-acetate (PMA), an activator of the $Ca^{++}$/diacylglycerol-dependent protein kinase C, and/or the $Ca^{++}$ ionophore A23187 had no effect on the NPY mRNA content in the absence of elevated cAMP. However, the combination of PMA and foskolin markedly increased the NPY mRNA content. Combined treatment with Dex, forskolin, PMA, and A23187 resulted in a 50-200-fold elevation of NPY mRNA. The results demonstrate synergistic effects of the four effectors in stimulating NPY gene expression.

Treatment of PC12 cells with NGF for 48 h resulted in a 4-fold elevation of the NPY mRNA abundance.

Glucocorticoids and cAMP synergistically increase the abundance of pEnk mRNA in C6 rat glioma cells. The mechanism of this increase was studied by run-on transcription experiments involving nuclei isolated from C6 cells treated with or without Dex and/or forskolin for 1-24 h. Labeled run-on transcripts were hybridized with excess pEnk and beta-actin cDNAs, and relative pEnk transcription rates were determined. Dex alone had no effect on this rate, and forskolin alone elicited a brief stimulation that reached 6-fold at 1 h but disappeared by 4 h. Dex + forskolin elicited a more sustained stimulation of 5-6 fold at 2-6 h and 3-fold at 24 hours. Inhibition of protein synthesis by cycloheximide did not affect basal or stimulated pEnk transcription, indicating that _de novo_ synthesis of protein factor(s) is not required. Dex did not alter the basal or forskolin-stimulated cAMP content of C6 cells. These results suggest that cAMP elevates pEnk mRNA in C6 cells by stimulating transcription and that glucocorticoids exert a permissive effect by sustaining the rate of cAMP-stimulated transcription.

Hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)$P_2$) produces two prospective intracellular messengers: inositol-1,4,5-trisphosphate ($InsP_3$), which releases $Ca^{2+}$ from intracellular stores; and diacylglycerol (DG), which with $Ca^{2+}$ activates protein kinase C. The formation of these two substances triggered by one external messenger, bradykinin, leads to the appearance of two different sequential membrane conductance changes in NG108-15 neuroblastoma-glioma hybrid cells. The addition of bradykinin to these cells rapidly stimulates hydrolysis of PtdIns(4,5)$P_2$ to $InsP_3$ and DG, raises intracellular $Ca^{2+}$, and hyperpolarizes and then depolarizes the cell membrane. By voltage-clamp recording hyperpolarization was shown to result from the activation of a pharmacologically-identifiable species of $Ca^{2+}$-dependent $K^+$ current. This is also activated by intracellular injections of $Ca^{2+}$ or $InsP_3$ so may be attributed to the formation and action of $InsP_3$. The subsequent depolarization results primarily from the inhibition of a different, voltage-dependent $K^+$ current, the M-current that is also inhibited by DG activators. Hence a dual, time-dependent role is described for these two intracellular messengers in the control of neuronal signalling by a peptide.

Iontophoretic injections of inositol 1,4,5-trisphosphate inside NG108-15 cells evoked an outward $K^+$ current across the outer cell membrane, probably activated by the release of intracellular $Ca^{2+}$. No such current was produced by equivalent intracellular injections of inositol 1,3,4-trisphosphate or inositol

1,3,4,5-tetrakisphosphate. Instead, these compounds evoked an inward current with a reversal potential of about -20 mV, which may therefore be due to a non-specific cation conductance. This suggests that the latter compounds are unable to release sufficient $Ca^{2+}$ to activate the $Ca^{2+}$ -dependent $K^+$ current in these cells.

The role of inositol 1,4,5-trisphosphate and diacylglycerol as possible mediators of the membrane current responses of NG108-15 cells to bradykinin (BK) has been tested using intracellular iontophoresis of $InsP_3$ and external application of phorbol dibutyrate (PDBu) and 1-oleoyl-2-acetylglycerol (DAG). Intracellular iontophoresis of $InsP_3$ into cells clamped at -30 to -50 mV produced (i) a transient outward current, (ii) a transient outward current followed by an inward current, or (iii) an inward current. All currents were accompanied by an increased input conductance. The transient outward current reversed at between -80 and -90 mV. The reversal potential was shifted to more positive potentials on raising extracellular $[K^+]$, suggesting that it resulted from an increased $K^+$ -conductance. The outward current was inhibited by apamin (0.4 uM) or d-tubocurarine (0.2-0.5 mM); these drugs also inhibit the outward current produced by BK or by intracellular $Ca^{2+}$ injections. The outward current was also slowly reduced in the absence of external $Ca^{2+}$ or in the presence of a solution containing 0.5 mM $Cd^{2+}$ and 2 mM $Co^{2}$. Iontophoretic injection of inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate, guanosine trisphosphate or inorganic phosphate did not evoke an outward current but produced only an inward current with an increased conductance, reversing at between -10 and -20 mV. Bath-application of PDBu (10 nM - 1 uM) or DAG (1 - 10 µM) produced an inward current with a fall in input conductance. The inward current was voltage-dependent and was accompanied by an inhibition of the time-dependent current relaxations associated with activation or deactivation of the voltage-dependent $K^+$-current. PDBu did not clearly reduce the $Ca^{2+}$-current or the $Ca^{2+}$-dependent $K^+$-current recorded in these cells. During superfusion with PDBu, the outward current produced by intracellular iontophoresis of $InsP_3$ was greatly enhanced. Analysis of homogenates of $^{32}P$-labelled NG108-15 cells by two-dimensional gel electrophoresis revealed more than 260 phosphoproteins. Four proteins were phosphorylated due to the addition of both BK and PDBu, including the 80,000 $M_r$ substrate for protein kinase C. The results support the view that the two membrane current responses to BK might result from accelerated membrane phosphatidylinositide hydrolysis. One product, $InsP_3$, releases $Ca^{2+}$ and activates an apamin/curare sensitive outward $K^+$ current; this effect is imitated by intracellular $InsP_3$ iontophoresis. The second product, DAG, activates protein kinase C, which inhibits the voltage-dependent $K^+$-current and generates an inward current; this effect is imitated by external application

of PDBu or DAG, and is associated with increased protein phosphorylation.

The action of bradykinin, inositol 1,4,5-trisphosphate, and phorbol dibutyrate on the release of acetylcholine (ACh) was studied electrophysiologically on short-distance (<20 μm) synapses formed between cultured NG108-15 cells and rat muscle cells. Intophoretic application of BK onto the somatic surface of an NG108-15 cell produced an increase in frequency of m.e.p.p.s for 40-50 sec in the paired myotube. Some m.e.p.p.s were evoked during BK-induced hyperpolarization (10-20 sec) of the hybrid cell soma. A few m.e.p.p.s also were elicited during BK-induced depolarization. Iontophoretic injection of $Ca^{2+}$ into an NG108-15 cell soma generated m.e.p.p.s for a very brief period (less than 3 sec), coincident with somatic hyperpolarization. No increase was observed during a subsequent somatic depolarization induced by a larger current of $Ca^{2+}$. Iontophoretic injection of $InsP_3$ into the cytoplasm of an NG108-15 cell soma transiently evoked m.e.p.p.s during the $InsP_3$-induced hyperpolarizing phase. A large $InsP_3$ injection caused sustained generation of m.e.p.p.s for 2-4 min, associated with $InsP_3$-evoked depolarization. Within 3-5 min after exposure of NG108-15-myotube pairs to 1 μM PDBu, the m.e.p.p. frequency increased by 2-5 times and reached a plateau after 8 min. The increase continued after washout of the drug. The PDBu-induced increase of m.e.p.p.s was still observed when the membrane potential of the NG108-15 cell was clamped at -30 mV. The data suggest that the BK-induced facilitation results from the action of two intracellular second messengers: an $InsP_3$-dependent release of $Ca^{2+}$ from the intracellular storage sites and protein phosphorylation catalyzed by DAG and $Ca^{2+}$ activated protein kinase C.

We have been investigating the induction of ACh receptor aggregation on muscle cells in culture, by soluble macromolecules from neurons. We previously observed a tendency for vinculin and α-actinin to be concentrated at the sites of newly formed ACh receptor aggregates. Quantitative studies carried out this year confirmed this observation. In addition, comparison of control and brain extract-treated myotubes indicate that the overall amount of vinculin and α-actinin is not substantially increased. Taken together, these results suggest that ACh receptor aggregates induced by brain extract tend to form at cell surface sites previously enriched in α-actinin and vinculin.

We previously demonstrated that the induction of the deposition of laminin and collagen type IV and formation of basal lamina on cultured myotubes by neural factors are sensitive to ascorbate oxidase. Since these experiments were carried out in the presence of horse serum, it was possible that the ascorbate required for these effects was derived from the serum itself. In experiments

using dialysed (ascorbate free) serum in the medium, we have shown that ascorbate or ascorbate-like factor is derived from the neural sources.

Until now, there has been no evidence for regulation of _E. coli_ adenylate cyclase by a GTP-binding protein. However, we found that the GTP-binding protein EF-Tu can stimulate _E. coli_ adenylate cyclase activity. Homogeneous EF-Tu specifically increased the activity of purified adenylate cyclase by as much as 70%; other _E. coli_ GTP-binding proteins had no effect on enzyme activity. A study of the guanine nucleotide specificity for EF-Tu-mediated stimulation of adenylate cyclase activity suggested that the preferred activator is Ef-Tu·GDP. To account for the GTP-specific stimulation of adenylate cyclase activity which we and others have observed in permeable cells, we propose that the nucleotide specificity of EF-Tu-dependent activation of adenylate cyclase is governed by other factors in the cell.

Inorganic orthophosphate is an important regulator of adenylate cyclase activity and substantially stimulates the activity of the enzyme provided the proteins of the PTS are present. The purpose of the study was to deduce which of the PTS proteins are necessary for the stimulation of adenylate cyclase activity by inorganic orthophosphate. We found that the stimulation of activity by Pi required the presence of all the PTS proteins, but that the presence of IIIglc in the absence of the other PTS proteins resulted in a Pi-dependent inhibition of adenylate cyclase activity. This unexpected finding strongly suggests that IIIglc can interact with adenylate cyclase in the absence of Enzyme I. The ramifications of this observation are that the mechanism for adenylate cyclase regulation may involve the decomposition of an inhibited form of the enzyme that results from interaction with IIIglc.

We found that three mutationally altered forms of the cAMP receptor protein can function independently of cAMP to stimulate transcription in an _in vitro_ transcription system. The explanation for the nucleotide-independent activity of these proteins is that they assume a conformation similar to that of the wild-type protein when it is complexed to cAMP. This conclusion came from proteolysis susceptibility studies on the wild-type and mutant proteins. It was found that some, but not all, of the mutant proteins showed an inhibitory effect of spermidine on their transcription activity. This unique effect of spermidine was correlated with the _in vivo_ behavior of the proteins with respect to glucose-mediated repression. These studies suggest that spermidine may affect promoter recognition for cAMP receptor proteins with poor affinity for promoters but not those with high promoter affinity. Therefore, modulation of the effective levels of spermidine may be an important physiological regulator of

transcription.

Polypeptides rich in lysine markedly stimulate the phosphorylation of some membrane proteins catalyzed by a protein kinase in *Xenopus* oocyte membranes or membranes from NG108-15 neuroblastoma-glioma hybrid cells. A synthetic peptide containing the last 14 amino acid residues of human Ki-ras 2 protein (KKKKKKSKTKCVIM), which is rich in lysyl-residues, also stimulates protein phosphorylation. These effects were not observed with polyarginine. Polylysine peptides including the synthetic c-Ki-ras 2 peptide also stimulate the *in vitro* phosphorylation of membrane inositolphospholipids, resulting in the synthesis primarily of phosphatidylinositol 4-phosphate and to a lesser extent, phosphatidylinositol 4,5-bisphosphate.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00009-13 LBG

PERIOD COVERED

October 1, 1986 - September 30, 1987

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
Hemin Chin, Staff Fellow, LBG, NHLBI
Patricia Bray, Biologist, LBG, NHLBI
Li-Shan Hsieh, Visiting Fellow, LBG, NHLBI
Wu-Hong Tsai, Visiting Fellow
Maria Giovanni, Staff Fellow, NLBG, NHLBI
David Trisler, Guest Worker, LBG, NHLBI
Haruhiro Higashida, Visiting Scientist, LBG, NHLBI

COOPERATING UNITS (if any)

Gerald Aurbach, Chief, MDB, NIADDK
Allen Spiegel, MDB, NIADDK
Bruce Schrier, LDN, NICHD
Lou Hirsch, Dept. of Biochem., U. of Texas, Dallas, Texas

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section of Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 11 | 9 | 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.)

Mouse neuroblastoma-rat retina hybrid cell lines were established to rescue the expression of retinal genes. A monoclonal antibody that binds to cells from one hybrid line recognizes an antigen expressed by few cell types in retina and brain. cDNA corresponding to the neural antigen was cloned and sequenced. Additional monoclonal antibodies were obtained that bind to antigens that are markers of cell types in retina. Clones for 4 species of $\alpha_s$ cDNA and 1 $\alpha_s$ genomic DNA were obtained that correspond to the $\alpha_s$ subunit of $G_s$ signal transduction protein. Two $\alpha_i$-1 cDNA clones were obtained and sequenced. In addition, DNA clones for rat and human $\alpha_o$ genes were obtained. Monospecific antibodies to an $\alpha$-subunit of voltage-sensitive $Ca^{2+}$ channels from rat T-tubules were shown to activate voltage-sensitive calcium channels of parathyroid cells. Both cDNA and genomic DNA clones were obtained that correspond to species of poly $A^+$ RNA that increase or decrease in abundance when cells are treated with dibutyryl cAMP. TOP, a cell membrane protein that is distributed in a dorsal-ventral topographic gradient in chick retina was shown to be distributed in an inverted but matching ventral-dorsal gradient in chick embryo tectum. Proteins rich in lysine markedly stimulate the activity of a protein kinase in _Xenopus_ oocyte membranes and NG108-15 membranes. NG108-15 cells respond to bradykinin by increased hydrolysis of phosphatidylinositol-4,5-bisphosphate and accumulation of inositol-1,4,5-trisphosphate, which releases $Ca^{2+}$ from intracellular stores, and diacylglycerol, which activates protein kinase C. Cytoplasmic $Ca^{2+}$ activates a $Ca^{2+}$-dependent $K^+$ channel leading to cell hyperpolarization. The activation of protein kinase C by bradykinin leads to an increase in the phosphorylation of 4 proteins and the inhibition of M-channels. Activation of protein kinase C greatly potentiates $Ca^{2+}$ dependant acetylcholine secretion from NG108-15 cells.

Project No. Z01 HL 00009-13 LBG

Major Findings:

N18TG-2 mouse neuroblastoma cells were fused with 18-day rat embryo retina cells, and somatic cell hybrid lines then were established to obtain clonal cell lines that continue to express genes characteristic of cells in retina. One of the hybrid cell lines generated, N18RE-103 possesses high tyrosine hydroxylase activity and synthesizes dopamine; whereas, tyrosine hydroxylase is not expressed by the parental N18TG-2 cells. N18RE-103 cells also possess voltage-sensitive $Na^+$, $K^+$, and $Ca^{2+}$ channels. N18RE-103 cells were used as an immunogen for the production of hybridoma cell lines that synthesize monoclonal antibodies to antigens expressed by N18RE-103 cells and retina. Thirty-three of the 374 hybridoma cell lines obtained synthesize antibodies to antigens expressed by N18RE-103 cells and rat retina cells, but not with cell lines from other tissues. Some of the antigens recognized by the monoclonal antibodies are expressed by different cell types in adult rat retina. For example, antibody 41C5 binds to antigen expressed by some, but not all, retinal ganglion neurons. Antibody 41A4 binds to antigen associated with the ganglion neuron layer. Antibody 89A5 binds to molecules restricted predominantly to the outer segments of photoreceptor cells. Antibody 38B6 binds to an abundant antigen on photoreceptor cell bodies and to less abundant antigen associated with horizontal, bipolar, and amacrine neuron soma. In contrast, antibody 35F8 recognizes antigen distributed in a punctate manner in the inner nuclear layer, the inner synaptic layer, and ganglion neuron layer of retina.

A λgt11 cDNA expression library was prepared and the recombinants were screened with antibody 41C5. One positive recombinant clone, λ41C5, was detected, which directs the synthesis of a 41C5-β galactosidase fusion protein recognized by antibody 41C5. Northern blot analysis with N18RE-103 poly $A^+$ RNA revealed one species of 41C5 poly $A^+$ RNA with a chain length of approximately 1 Kb. The nucleotide sequence of the λ41C5 insert was determined. One open reading frame was found, but no homology was detected between the predicted amino acid sequence of 41C5 and other proteins.

The distribution of 41C5 protein was shown by indirect immunofluorescence to be highly restricted to certain cells in rat retina and brain; the antigen was not detected in striated muscle, cardiac muscle, liver, or kidney. The distribution of 41C5 mRNA also was shown by *in situ* hybridization with a synthetic labeled oligodeoxynucleotide probe to be highly restricted in rat retina

and brain. 41C5 cDNA will be used as a probe to study the mechanisms that enable only a few cell types in the nervous system to express the 41C5 gene.

Hybridoma cell lines were generated that synthesize antibodies directed against cells from rat or rabbit retina that were used as immunogens. One monoclonal antibody detects an antigen that is most abundant in the outer layer of axons in the optic nerve. An antibody specific for the inner and outer synaptic layers of the retina was found as well as antibodies that bind only to Muller cells. Still other antibodies are specific for astrocytes in the retinal ganglion neuron layer. Some of the antigens that are markers of cell types were found to be proteins and were partially purified.

As described previously, 4 species of $\alpha_s$ cDNA clones were found that differ in nucleotide sequence in the region that corresponds to amino acid residues 71-88 in $\alpha_s$ protein, a subunit of $\alpha_s$ signal transduction protein. A mechanism was proposed for generating 4 species of $\alpha_s$ mRNA from a common precursor RNA transcribed from a single gene by 2 types of alternative splicing. Further work on $\alpha_s$ mRNA revealed that half the normal amount of $\alpha_s$ mRNA is present in fibroblasts of pseudo- hypoparathyroid patients. Levels of $\alpha_s$ mRNAs that code for low and high molecular weight forms of $\alpha_s$ protein were reduced with no apparent change in the ratio of the different species of $\alpha_s$ mRNA to one another. These results suggest that the genetic lesion in pseudohypoparathyroidism type Ia either decreases the rate of synthesis of a common precursor of the 4 species of $\alpha_s$ mRNA or increases equally the rates of turnover of the four species of $\alpha_s$ mRNA.

Two $\alpha_i$-1 cDNA clones also were obtained from a human brain cDNA library that correspond to $\alpha_i$ protein subunits of $G_i$ signal-transduction proteins. One of the cDNA inserts was sequenced completely, the other partially. The nucleotide sequence of human brain $\alpha_i$-1 is similar to that of bovine brain $\alpha_i$-1, but differs significantly from $\alpha_i$-2 cDNAs from human monocytes, rat glioma cells, and mouse macrophages in nucleotide and amino acid sequences (88% and approximately 73% homology, respectively). The 3'-untranslated region of human brain $\alpha_i$-1 also differs markedly from that of human monocyte $\alpha_i$-2.

Five genomic DNA clones for $\alpha$ genes were obtained. One clone was identified as a gene for $\alpha_s$, and 2 clones, 1 from a human genomic DNA library, the other from a rat genomic DNA library, were identified as $\alpha_o$ genes. Further work is needed to identify the 2 remaining $\alpha$ genomic DNA clones. The focus in current

studies is to define the nucleotide sequences of the 5'-upstream regulatory regions of α genes.

A λgt11 cDNA library was constructed from poly $A^+$ RNA from 14 day chick embryo retina and screened for recombinants with antibodies to β-subunits. One recombinant was detected with 9 out of 10 monospecific rabbit antibody preparations directed against the β-subunit of bovine transducin. The nucleotide sequence of the cDNA insert was determined and the predicted amino acid sequence was compared to that of the bovine β-subunit. Only a small region of homology was observed; hence, the cloned DNA is not related to the β Six of 10 amino acid residues predicted for the recombinant DNA were identical to amino acid residues near the N-terminus of the β-subunit and 2 glutamine residues of the β-subunit were replaced by glutamic acid residues. A synthetic decapeptide corresponding to the amino-terminal residues of β blocked the binding of anti β antibodies to β-subunits. These results show that the amino terminal decapeptide of the β- subunit is a major antigenic site of the native protein.

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The half-life of TOP in cultured retinal cells in the presence of cycloheximide or actinomycin D was 5 or 6 hr, respectively. Ablation of cells at the poles of the gradient in 60 hr chick embryos altered TOP expression during subsequent retinal development. Cells at the dorsal pole of the 13-day embryo retinas, 11.5 days after dorsal ablation, expressed 50% less TOP than normal and those at the ventral pole, after ventral ablation, expressed 300% more TOP than normal. Cells in other regions of the retina expressed normal levels of TOP.

Polypeptides rich in lysine markedly stimulate the phosphorylation of some membrane proteins catalyzed by a protein kinase in *Xenopus* oocyte membranes or membranes from NG108-15 neuroblastoma-glioma hybrid cells. A synthetic peptide containing the last 14 amino acid residues of human Ki-ras 2 protein (KKKKKKSKTKCVIM), which is rich in lysyl-residues, also stimulates protein phosphorylation. These effects were not observed with polyarginine. Polylysine peptides including the synthetic c-Ki-ras 2 peptide also stimulate the *in vitro* phosphorylation of membrane inositolphospholipids, resulting in the synthesis primarily of phosphatidylinositol 4-phosphate and to a lesser extent, phosphatidylinositol 4,5-bisphosphate.

Hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)$P_2$) produces two prospective intracellular messengers: inositol-1,4,5-trisphosphate ($InsP_3$), which releases $Ca^{2+}$ from intracellular stores; and diacylglycerol (DG), which activates protein kinase C. The formation of these two substances triggered by one external messenger, bradykinin, leads to the appearance of two different sequential membrane conductance changes in NG108-15 neuroblastoma-glioma hybrid cells. The addition of bradykinin to these cells rapidly stimulates hydrolysis of PtdIns(4,5)$P_2$ to $InsP_3$ and DG, raises intracellular $Ca^{2+}$ and hyperpolarizes and then depolarizes the cell membrane. By voltage-clamp recording hyperpolarization was shown to result from the activation of a pharmacologically-identifiable species of $Ca^{2+}$-dependent $K^+$ current. This is also activated by intracellular injections of $Ca^{2+}$ or $InsP_3$ so may be attributed to the formation and action of $InsP_3$. The subsequent depolarization results primarily from the inhibition of a different, voltage-dependent $K^+$ current, the M-current that is also inhibited by DG activators. Hence a dual, time-dependent role is described for these two intracellular messengers in the control of neuronal signalling by a peptide.

Iontophoretic injections of inositol 1,4,5-trisphosphate inside NG108-15 cells evoked an outward $K^+$ current across the outer cell membrane, probably activated by the release of intracellular $Ca^{2+}$. No such current was produced by equivalent intracellular injections of inositol 1,3,4-trisphosphate or inositol 1,3,4,5-tetrakisphosphate. Instead, these compounds evoked an inward current with a reversal potential of about -20 mV, which may therefore be due to a non-specific cation conductance. This suggests that these compounds are unable to release sufficient $Ca^{2+}$ to activate the $Ca^{2+}$ -dependent $K^+$ current in these cells.

The role of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) as possible mediators of the membrane current

responses of NG108-15 cells to bradykinin (BK) has been tested using intracellular iontophoresis of $InsP_3$ and external application of phorbol dibutyrate (PDBu) and 1-oleoyl-2-acetylglycerol (DAG). Intracellular iontophoresis of $InsP_3$ into cells clamped at -30 to -50 mV produced (i) a transient outward current, (ii) a transient outward current followed by an inward current, or (iii) an inward current. All currents were accompanied by an increased input conductance. The transient outward current reversed at between -80 and -90 mV. The reversal potential was shifted to more positive potentials on raising extracellular $[K^+]$, suggesting that it resulted from an increased $K^+$ -conductance. The outward current was inhibited by apamin (0.4 uM) or d-tubocurarine (0.2-0.5 mM); these drugs also inhibit the outward current produced by BK or by intracellular $Ca^{2+}$ injections. The outward current was also slowly reduced in the absence of external $Ca^{2+}$ or in the presence of a solution containing 0.5 mM $Cd^{2+}$ and 2 mM $Co^{2}$. Iontophoretic injection of inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate, guanosine trisphosphate or inorganic phosphate did not evoke an outward current but produced only an inward current with an increased conductance, reversing at between -10 and -20 mV. Bath-application of PDBu (10 nM - 1 uM) or DAG (1 - 10 µM) produced an inward current with a fall in input conductance. The inward current was voltage-dependent and was accompanied by an inhibition of the time-dependent current relaxations associated with activation or deactivation of the voltage-dependent $K^+$-current. PDBu did not clearly reduce the $Ca^{2+}$-current or the $Ca^{2+}$-dependent $K^+$-current recorded in these cells. During superfusion with PDBu, the outward current produced by intracellular iontophoresis of $InsP_3$ was greatly enhanced. Analysis of homogenates of $^{32}P$-labelled NG108-15 cells by two-dimensional gel electrophoresis revealed more than 260 phosphoproteins. Four proteins were phosphorylated due to the addition of both BK and PDBu, including the 80,000 $M_r$ substrate for protein kinase C. The results support the view that the two membrane current responses to BK might result from accelerated membrane phosphatidylinositide hydrolysis. One product, $InsP_3$, releases $Ca^{2+}$ and activates an apamin/curare sensitive outward $K^+$ current; this effect is imitated by intracellular $InsP_3$ iontophoresis. The second product, DAG, activates protein kinase C to inhibit the voltage-dependent $K^+$-current and generate an inward current; this effect is imitated by external application of PDBu or DAG, and is associated with increased protein phosphorylation.

The action of bradykinin (BK), inositol 1,4,5-trisphosphate ($InsP_3$), and phorbol dibutyrate (PDBu) on the release of acetylcholine (ACh) was studied electrophysiologically on

short-distance (<20 μm) synapses formed between cultured NG108-15 cells and rat muscle cells. Intophoretic application of BK onto the somatic surface of an NG108-15 cell produced an increase in frequency of m.e.p.p.s for 40-50 s in the paired myotube. Some m.e.p.p.s were evoked during BK-induced hyperpolarization (10-20 sec) of the hybrid cell soma. A few m.e.p.p.s also were elicited during BK-induced depolarization. Iontophoretic injection of $Ca^{2+}$ into an NG108-15 cell soma generated m.e.p.p.s for a very brief period (less than 3 sec), coincident with somatic hyperpolarization. No increase was observed during a subsequent somatic depolarization induced by a larger current of $Ca^{2+}$. Iontophoretic injection of $InsP_3$ into the cytoplasm of an NG108-15 cell soma transiently evoked m.e.p.p.s during the $InsP_3$-induced hyperpolarizing phase. A large $InsP_3$ injection caused sustained generation of m.e.p.p.s for 2-4 min, associated with $InsP_3$-evoked depolarization. Within 3-5 min after exposure of NG108-15-myotube pairs to 1 μM PDBu, the m.e.p.p. frequency increased by 2-5 times and reached a plateau after 8 min. The increase continued after washout of the drug. The PDBu-induced increase of m.e.p.p.s was still observed when the menmbrane potential of the NG108-15 cell was clamped at -30 mV. The data suggest that the BK-induced facilitation results from the action of two intracellular second messengers: an $InsP_3$-dependent release of $Ca^{2+}$ from the intracellular storage sites and protein phosphorylation by DAG-activated protein kinase C.

Publications:

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2. Bray, P., Carter, A., Guo, V., Puckett, C., Kamholz, J., Spiegel, A., and Nirenberg, M.: Human cDNA Clones for an α Subunit of $G_i$ Signal-transduction Protein. Proc. Natl. Acad. Sci., USA 84, 5115-5119 (1987).

3. Carter, A., Bardin, C., Collins, R., Simons, C., Bray, P. and Spiegel, A.: Reduced Expression of Multiple Forms of $G_s$ alpha in Pseudohypoparathyroidism Type Ia. Proc. Natl. Acad. Sci., USA, In Press.

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324-328 (1987).

5. Trisler, D. and Collins, F.: Corresponding Spatial Gradients of TOP Molecules in the Developing Retina and Optic Tectum. Science, In Press.

6. Trisler, D.: Synapse Formation in Retina is Influenced by Molecules that Identify Cell Position. Current Topics in Devel. Biol. 21, 277-308 (1987).

7. Trisler, D.: Molecular Markers of Cell Position in Avian Retina are Involved in Synapse Formation. American Zoologist 27, 189-206 (1987).

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11. Brown, D.A. and Higashida, H.: Membrane Current Responses of NG108-15 Mouse Neuroblastoma x Rat Glioma Hybrid Cells to Bradykinin. J. Physiol., In Press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL00017-12 LBG

PERIOD COVERED

October 1, 1986 - September 30, 1987

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
Zhao-Yong Xi, Special Volunteer, LBG-NHLBI
Bernhard E. Flucher, Special Volunteer, LBG-NHLBI

COOPERATING UNITS (if any)

R. Bloch, University of Maryland Medical School, Dept. of Physiology
Z. Vogel, The Weizmann Institute of Science, Neurobiology Dept.

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3 | 1 | 2 |

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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 acetylchone receptors and contribute to the assembly of a postsynaptic transmembrane complex. 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 stabilizatin or elimination of aggregates which occur in developing neuromuscular junctions.

We use fluorescence and electron microscopy, as well as immunocytochemistry, to follow changes in the distribution of acetylcholine receptors and associated cell surface components, and to study the underlying ultrastructural changes.

In the past year, we have found that:

1) Brain extract-induced acetylcholine receptor aggregates may form in regions of the cell surface previously enriched in the proteins vinculin and α-actinin.

2) The deposition of collagen and laminin, and the formation of muscle cell basal lamina induced by embryonic brain extract and ciliary ganglion are dependent on ascorbic acid or an ascorbate-like factor present in these neural preparations.

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 and contribute to the assembly of a postsynaptic transmembrane complex. 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 elimnated.

Methods:

We stain rat skeletal myotubes grown in monolayer culture with rhodamine-labeled α-bungarototoxin (α-BT) in order to visualize ACh receptor sites with the fluorescence microscope.

The distribution of basement membrane and cytoskeletal proteins in the cultures has been determined by indirect immunofluorescence, using antisera, affinity purified antibodies, and monoclonal antibodies against purified proteins. The staining is done on intact cultures, on cryostat sections of pelleted cultures and on cell surface fragments isolated from the cultures. Techniques for colloidal gold-antibody staining of plastic sections and ultrathin cryosections are now being adapted for electron microscopic immunocytochemical studies.

ACh receptor aggregating material is routinely prepared from extracts of fetal rat or pig brain.

Cell ultrastructure is studied in thin sections of Epon embedded cultures.

Major Findings:

1. Cytoplasmic components of ACh receptor aggregates - We previously observed a tendency for vinculin and α-actinin to be concentrated at the sites of newly formed ACh receptor aggregates. Quantitative studies carried out this year confirmed this observation. In addition, comparison of control and brain extract-treated myotubes indicated that the overall amount of vinculin and α-actinin was not substantially increased. Taken together, these results suggest that ACh receptor aggregates induced by brain extract tend to form at cell surface sites previously enriched in α-actinin and vinculin.

2. Involvement of ascorbate in extracellular matrix formation. We previously demonstrated that the induction of the deposition of laminin and collagen type IV and formation of basal lamina on cultured myotubes by neural factors are sensitive to ascorbate oxidase. Since these experiments were carried out in the presence of horse serum, it was possible that the ascorbate required for

these effects was derived from the serum itself. In experiments using dialysed (ascorbate free) serum in the medium, we have shown that ascorbate or ascorbate-like factor is derived from the neural sources.

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.

Publications:


1. Daniels, M.P., Krikorian, J.G., Ling, A. and Olek, A.J.: Neural factor induced formation of acetylcholine receptor aggregates on myotubes: An *in vitro* model for development of the postsynaptic cell surface complex. In Vernadakis, A., Lauder, J., Privat, A., Timiras, P. and Giacobini, E. (Eds): *Model Systems of Development and Aging of the Nervous System*. Martinus Nijhoff Publishers, Boston, 1987.

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3. Olek, A.J., Krikorian, J.G., and Daniels, M.P.: Early stages in the formation and stabilization of acetylcholine receptor aggregates on cultured myotubes: Sensitivity to temperature and azide. *Devel. Biol.* 117:24-34, 1986.

4. Vogel, Z., Daniels, M.P., Chen, T., Xi, Z.Y., Bachar, E., Ben David, L., Rosenberg, N., Krause, Michael, Duksin, D. and Kalcheim, C: Ascorbate-like factor from embryonic brain: Role in collagen formation, basement membrane deposition, and acetylcholine receptor aggregation by muscle cells. *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 00018-10 LBG

PERIOD COVERED
October 1, 1986 - September 30, 1987

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

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

| | | | |
|---|---|---|---|
| PI: | Steven L. Sabol | Medical Officer (Research) | LBG, NHLBI |
| Others: | Jay Joshi | Senior Staff Fellow | LBG, NHLBI |
| | Hiroshi Higuchi | Visiting Fellow | LBG, NHLBI |

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.3 | 3.0 | 0.3 (summer student) |

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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.)*


This project consists of several studies on the molecular genetics of protein precursors of neuropeptides in the mammalian nervous system.

One study concerns the regulation of the gene coding for neuropeptide Y (NPY), an important regulator in the central and peripheral nervous systems. The primary structure of the rat NPY precursor was determined by sequencing of rat brain cDNA clones. The precursor contains the NPY sequence and a COOH-terminal peptide sequence separated by a proteolytic processing site. In comparison with the human precursor, the strong evolutionary conservation of both peptide sequences (93-100%) suggests that both peptides are physiologically important. In the rat central nervous system, NPY mRNA (800 bases) is most abundant in the striatum and cortex, followed by hippocampus, hypothalamus, and spinal cord. The rat adrenal, spleen, heart, and lung have significant levels of NPY mRNA. In PC12 rat pheochromocytoma cells, the abundance of PNY mRNA was increased by treatment with glucocorticoids, cAMP elevation, or nerve growth factor. Phorbol esters that activate protein kinase C also elevate NPY mRNA but only when the cAMP level is concomitantly elevated. Thus, the NPY gene is regulated by multiple effectors, some acting cooperatively.

A second project concerns regulation of transcription of the gene coding for proenkephalin, the precursor of the opioid peptides methionine- and leucine-enkephalin. Glucocorticoids and cAMP synergistically increase the transcription of the proenkephalin gene and the abundance of proenkephalin mRNA in C6 rat glioma cells. Detailed nuclear run-on transcription experiments demonstrated that glucocorticoids exert a permissive effect on proenkephalin gene transcription by prolonging the transcriptional stimulation elicited by cAMP elevation.

These studies shed light on the control of biosynthesis of peptides that are important in autonomic regulation and possibly higher cognitive function.

## Project Description

### Objectives

A. Neuropeptide Y (NPY), is abundantly distributed in the central and peripheral nervous systems as well as in adrenal chromaffin cells. Evidence is mounting that NPY is involved in autonomic regulation of such processes as peripheral and cardiac artery blood pressure, circadian rhythms, release of hypothalamic hormones, and feeding behavior. The abundance of NPY in cognitive centers in the brain suggests that it may also be involved in higher brain functions. When this project was started 1 1/2 years ago, little was known about the NPY gene and its regulation. To study such regulation in experimental systems, generally of rodent origin, we sought to obtain a sensitive cDNA probe and clonal cell lines that express the NPY gene, then to study the regulation of NPY gene expression by hormones and second messengers.

B. The pentapeptides methionine-enkephalin and leucine-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 are currently studying the regulation of expression of the gene for the precursor (pre)proenkephalin (pEnk) in simple systems such as clonal neural cell lines. Knowledge about the control of pEnk gene regulation will help in understanding endogenous mechanisms of pain perception and neural control.

### Methods Employed

Messenger RNA purification, RNA and DNA blot hybridization analysis, cDNA cloning, colony and plaque hybridization with oligonucleotides and nick-translated probes, DNA sequencing, preparation of RNA (sense or antisense strand) from plasmids having RNA polymerase promoters, nuclear run-on transcription assays, preparation of chimeric plasmids containing regulatory elements and a reporter gene, transient gene expression assays, cell culture.

### Major Findings

A. Regulation of rat neuropeptide Y gene expression:

We (Drs. Higuchi and Sabol) isolated four independent NPY cDNA clones from two rat brain cDNA libraries in the lambda-gt11 vector by hybridization with a human NPY cDNA probe kindly provided by Dr. Jack Dixon (Purdue University). These clones were sequenced and were found to contain at least 511 bp of rat prepro-NPY cDNA. The mRNA sequence revealed an open reading frame coding for a 98-amino acid gene product containing a signal peptide, followed by a 36-residue sequence identical to human NPY, followed by a proteolysis/amidation signal Gly-Lys-Arg, followed by a 30-residue COOH-terminal peptide sequence that differs from the human counterpart in only two positions. This high degree of peptide sequence conservation is consistent with important physiological activities of both NPY and COOH-terminal peptides, an interesting finding since no function or activity is currently known for the COOH-terminal peptide.

The abundances of NPY mRNA (700-800 bases in length) in rat brain regions were determined by Northern and dot-blot analyses. The values (pg NPY mRNA/ug total RNA) are as follows: striatum, 6.1; frontal cortex, 5.0; hippocampus,

2.1; hypothalamus, 2.0; spinal cord, 1.8; medulla, 1.1; midbrain, 0.6; and cerebellum, 0.4. NPY mRNA was also abundant in adrenal gland, spleen, and heart and was detectable in lung, skeletal muscle, stomach, and thyroid. The levels of immunoreactive NPY peptide were found by Drs. Higuchi and Yang to be generally parallel to NPY mRNA levels except for hypothalamus, which was richest in NPY peptide, and spleen, which has a low abundance of NPY peptide compared to its relatively high NPY mRNA level. The large amount of NPY mRNA in spleen suggest pro-NPY synthesis by immune cells; this possibility is being tested.

RNA from several well-studied rodent lines known to contain NPY were found to contain diverse levels of NPY mRNA. PC12 rat pheochromocytoma cells and N18TG-2 mouse neuroblastoma cells possess relatively low basal levels of NPY mRNA (0.25 and 0.13 pg/ug total RNA), while NG108-15 mouse neuroblastoma x rat glioma hybrid cells contain remarkably high amounts of NPY mRNA (11 pg/ug RNA).

We have initiated a study of the effects of hormones and second messenger systems such as cyclic nucleotides and calcium ions on NPY gene expression. The results, some preliminary, are as follows:

Glucocorticoids and cyclic nucleotides: Treatment of PC12 cells with dexamethasone (Dex) for 24-96 hr increased the NPY mRNA abundance 2-5-fold. Elevation of the cAMP level with forskolin, or treatment with 8-bromo-cAMP, elicited a rapid but transient increase in the NPY mRNA level in PC12 cells, which was maximally 2.7-7 times the control at 8 h. In N18TG-2 cells, Dex or forskolin alone elicited only slight increases (50%) in NPY mRNA, but the combination of both drugs produced 4-7-fold elevations. In NG108-15 cells, glucocorticoids increased NPY mRNA, but cAMP elevation markedly and persistently reduced the high basal NPY mRNA abundance by 85%. Cyclic GMP and non-glucocorticoid steroids had no effect.

Calcium ion: Treatment of PC12 cells with phorbol 12-myristate 13-acetate (PMA), an activator of the $Ca^{++}$/diacylglycerol-dependent protein kinase C, and/or the $Ca^{++}$ ionophore A23187 had no effect on the NPY mRNA content in the absence of elevated cAMP. However, the combination of PMA and forskolin markedly and synergistically increased the NPY mRNA content. Combined treatment with Dex, forskolin, PMA, and A23187 produced 50-200-fold elevations of NPY mRNA. The results demonstrate cooperative effects of these four effectors in stimulating NPY gene expression.

Nerve growth factor (NGF): Treatment of PC12 cells with NGF for 48 h resulted in a 4-fold elevation of the NPY mRNA abundance.

We plan to investigate the mechanisms of action of these compounds by nuclear run-on transcription and DNA transformation/transient expression assays.

B. Regulation of proenkephalin (pEnk) gene expression.

Glucocorticoids and cAMP synergistically increase the abundance of pEnk mRNA in C6 rat glioma cells. We (Drs. Joshi and Sabol) examined the mechanism of this increase by run-on transcription experiments involving nuclei isolated from C6 cells treated with or without Dex and/or forskolin for 1-24 h. Labeled run-on transcripts were hybridized with excess pEnk and beta-actin cDNAs, and relative pEnk transcription rates were determined. Dex alone had no effect on this rate, and forskolin alone elicited a brief stimulation that reached 6-fold at 1 h but disappeared by 4 h. Dex + forskolin elicited a more sustained stimulation of 5-6 fold at 2-6 h and 3-fold at 24 h. Inhibition of protein synthesis by cycloheximide did not affect basal or stimulated pEnk transcription,

indicating that de novo synthesis of protein factor(s) is not required. Dex did not alter the basal or forskolin-stimulated cAMP content of C6 cells. These results suggest that cAMP elevates pEnk mRNA in C6 cells by stimulating transcription and that glucocorticoids exert a permissive effect by sustaining the rate of cAMP-stimulated transcription.

The glucocorticoid regulatory region of the pEnk gene is being localized by transient expression assays of plasmids having portions of the pEnk gene linked to a reporter gene. The mechanism of the synergism between glucocorticoids and cAMP also will be examined by such transient expression assays. The nature of cell-type specific expression of the pEnk gene is being examined (Dr. Higuchi and Dr. Sabol).

Publications

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2. Kanamatsu, T., Obie, J., Grimes, L, McGinty, J.F., Yoshikawa, K., Sabol, S., and Hong, J.S.: Kainic acid alters the metabolism of Met[5]-enkephalin and the level of dynorphin A in the rat hippocampus. J. Neuroscience, 6: 3094-3102.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00151-17 LBG

PERIOD COVERED

October 1, 1986 to September 30, 1987

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)

| | | | |
|---|---|---|---|
| PI: | A. Peterkofsky | Deputy Chief | LBG, NHLBI |
| Others: | E. Liberman | Staff Fellow | LBG, NHLBI |
| | J. Harman | Staff Fellow | LBG, NHLBI |
| | P. Reddy | Senior Staff Fellow | LBG, NHLBI |
| | J. Reizer | Senior Staff Fellow | LBG, NHLBI |

COOPERATING UNITS (if any) (1) N.Y. State Institute for Basic Research in Developmental Disabilities, Staten Island, N.Y. (D. Miller); (2) Laboratory of Molecular Genetics, NINCDS (K. McKenney); Dept. of Biology, Johns Hopkins University, Baltimore, MD. (D. Saffen, S. Roseman).

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Macromolecules Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5.2 | 4.2 | 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.)


A. Stimulation of Escherichia coli Adenylate Cyclase Activity by Elongation Factor Tu. A unique feature of eucaryotic adenylate cyclases is their interaction with GTP-binding proteins that mediate hormonal responses. We found that the most abundant protein in E. coli, the GTP-binding protein EF-Tu, which is important as an elongation factor in protein synthesis, also serves as a stimulator of adenylate cyclase activity.

B. Inhibition of E. coli Adenylate Cyclase Activity by Inorganic Orthophosphate Dependent on IIIglc of the Phosphoenolpyruvate:glycose Phosphotransferase System. The relationship of adenylate cyclase, inorganic orthophosphate and the proteins of the phosphoenolpyruvate:glycose phosphotransferase system (PTS) was studied. A strain deleted for the genes for Enzyme I and IIIglc of the PTS was transformed with plasmids expressing either Enzyme I and HPr, IIIglc or all three proteins. The fully reconstituted strain showed a Pi-dependent stimulation of adenylate cyclase activity; in contrast, the strain expressing only IIIglc showed a Pi-dependent inhibition of adenylate cyclase activity.

C. Structure-Function Analysis of cAMP-independent Forms of the cAMP Receptor Protein. The cAMP receptor protein ordinarily requires cAMP for function in operon expression. We examined the properties of three mutant forms of the protein which function in the absence of added cAMP. By cloning and DNA sequence analysis, we determined the primary structure of the mutant proteins. Additionally, we purified the mutant proteins and characterized the effect of these mutations on the secondary structure of the proteins. We also used a purified in vitro transcription system to study the properties of the proteins.

Project Description:

Objectives:

In Escherichia coli, gene expression depends on the availability and activity of both cAMP and the cAMP receptor protein. We have studied both the enzyme that makes cAMP (Adenylate Cyclase) as well as the protein that binds it (the cAMP receptor protein). Since eucaryotic forms of adenylate cyclase generally are regulated by proteins that bind GTP, one of the objectives of the work was to search for a GTP-binding protein that might regulate the activity of E. coli adenylate cyclase. Another aspect of the work dealt with the way in which inorganic orthophosphate regulates the activity of E. coli adenylate cyclase. Since it had previously been observed by us that the proteins of the sugar transport system known as the phosphoenolpyruvate:glycose phosphotransferase system (PTS) were essential for the inorganic orthophosphate-dependent stimulation of adenylate cyclase activity, an objective was to evaluate the reqirements for the isolated PTS proteins for this stimulatory effect. The cAMP receptor protein can be mutated to function in the absence of cAMP. The final objective of our studies was to characterize some of these mutant proteins by determining their primary structure, their conformations and their biological properties.

Methods Employed:

All studies were carried out with E. coli strains and proteins. Cloning of the gene for adenylate cyclase provided a basis for hyperexpression of the protein. The hyperexpressed protein was purified by conventional methodology to produce an enzyme that was about 10% pure. Adenylate cyclase activity was measured by procedures previously described. The effect of homogeneous EF-Tu (provided by Dr. Miller) on the enzyme activity was evaluated. Plasmids containing the genes for some or all of the proteins of the PTS were constructed and then inserted into a strain of E. coli deficient in these genes. The effect of the presence or absence of the PTS gene products on adenylate cyclase activity and its regulation by inorganic orthophosphate was tested in permeable cells. The genes for mutant forms of the cAMP receptor protein were cloned and sequenced to deduce the primary structures of the proteins. The genes were hyperexpressed to produce sufficient quantities of the proteins for biochemical studies. The proteins were purified to apparent homogeneity by conventional methods. Studies on the protease sensitivity of the proteins were done to evaluate their conformations in the absence and presence of added cyclic nucleotides. The reactivity under a variety of assay conditions of the purified proteins in an in vitro transcription system was also tested.

Major Findings:

A. Stimulation of Escherichia coli Adenylate Cyclase Activity by Elongation Factor Tu. Until now, there has been no evidence for regulation of E. coli adenylate cyclase by a GTP-binding protein. The present studies established that the GTP-binding protein EF-Tu can stimulate E. coli adenylate cyclase activity. Homogeneous EF-Tu specifically increased the activity of purified adenylate cyclase by as much as 70%; other E. coli GTP-binding proteins had no effect on enzyme activity. A study of the guanine nucleotide specificity for EF-Tu-mediated stimulation of adenylate cyclase activity suggested that the

preferred activator is Ef-Tu·GDP. To account for the GTP-specific stimulation of adenylate cyclase activity which we and others have observed in permeable cells, we propose that the nucleotide specificity of EF-Tu-dependent activation of adenylate cyclase is governed by other factors in the cell.

B. Inhibition of E. coli Adenylate Cyclase Activity by Inorganic Orthophosphate Dependent on IIIglc of the Phosphoenolpyruvate-glycose Phosphotransferase System. Inorganic orthophosphate is an important regulator of adenylate cyclase activity; it results in a substantial stimulation of the activity of the enzyme provided the proteins of the PTS are present. The purpose of the study was to deduce which of the PTS proteins are necessary for the stimulation of adenylate cyclase activity by inorganic orthophosphate. We found that the stimulation of activity by Pi required the presence of all the PTS proteins, but that the presence of IIIglc in the absence of the other PTS proteins resulted in a Pi-dependent inhibition of adenylate cyclase activity. This unexpected finding strongly suggests that IIIglc can interact with adenylate cyclase in the absence of Enzyme I. The ramifications of this observation are that the mechanism for adenylate cyclase regulation may involve the decomposition of an inhibited form of the enzyme that is constituted by an interaction with IIIglc.

C. Structure-Function Analysis of cAMP-independent Forms of the cAMP Receptor Protein. We found that three mutationally altered forms of the cAMP receptor protein can function independently of cAMP to stimulate transcription in an *in vitro* transcription system. The explanation for the nucleotide-independent activity of these proteins is that they assume a conformation similar to that of the wild-type protein when it is complexed to cAMP. This conclusion came from proteolysis susceptibility studies on the wild-type and mutant proteins. It was found that some, but not all, of the mutant proteins showed an inhibitory effect of spermidine on their transcription activity. This unique effect of spermidine was correlated with the *in vivo* behavior of the proteins with respect to glucose-mediated repression. These studies suggest that spermidine may affect promoter recognition for cAMP receptor proteins with poor affinity for promoters but not those with high promoter affinity. Therefore, modulation of the effective levels of spermidine may be an important physiological regulator of transcription.

Publications:


1. Reddy, P., Miller, D. and Peterkofsky, A.: Stimulation of *Escherichia coli* Adenylate Cyclase Activity by Elongation Factor Tu, a GTP-binding Protein Essential for Protein Synthesis. *J. Biol. Chem.* 261:11448-11451, 1986.

2. Harman, J. G., McKenney, K. and Peterkofsky, A.: Structure-Function Analysis of Three cAMP-independent Forms of the cAMP Receptor Protein. *J. Biol. Chem.* 261:16332-16339, 1986.

3. Liberman, E., Saffen, D., Roseman, S. and Peterkofsky, A.: Inhibition of *E. coli* Adenylate Cyclase Activity by Inorganic Orthophosphate is Dependent on IIIglc of the Phosphoenolpyruvate:glycose Phosphotransferase System. *Biochem. Biophys. Research Communs.* 141:1138-1144, 1986.

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## Role of Oxygen Mediated Inactivation of Enzymes in Protein Turnover, Aging, Neutrophil Function, and Oxygen Toxicity

Chronic and acute oxygen toxicity is implicated in a growing list of pathologic processes which include arthritis, the aging process, carcinogenesis, broncho-pulmonary dysplasia and adult respiratory distress syndrome, retinopathy of prematurity, and reperfusion-mediated ischemic damage. Oxidative modification of proteins has been demonstrated in some of these disorders and likely occurs in all of them. Oxidative modification might also function physiologically; for example, in controlling the switch from anaerobic to aerobic metabolism, in host defense mechanisms, in limitation of the inflammatory response, and in some mechanisms of protein turnover.

(a) Protein Turnover and the Age-related Accumulation of Altered Enzymes. In general, oxidatively modified proteins lose catalytic activity and become susceptible to proteolyitc degradation. Studies with bacterial glutamine synthetase have shown that its susceptibility to proteolysis correlates with the oxidation of the second of two histidine residues per subunit, and also with an increase in absorbance at 320 nm. Prolonged exposure of glutamine synthetase to an MFO system comprised of ascorbate, Fe(III) and $O_2$ does not lead to substantial fragmentation of the polypeptide chain; however, as the time of exposure increases there is a progressive increase in a multiplicity of protein species having lower isoelectric points. This is consistent with other studies showing that side chains of arginine, histidine and lysine residues in proteins are oxidized to carbonyl derivatives (presumably with loss of basic function).

(b) Oxygen Toxicity, Aging, and Exercise. To determine if the age-related accumulation of catalytically inactive or less active forms of enzymes is due to their oxidation by mixed-function oxidation (MFO) systems, the levels of oxidized proteins, inactive enzymes, and protease activities were determined in hepatocytes isolated from rats exposed to 100% oxygen, and from rats of various ages. The levels of oxidized protein increase with animal age and also with exposure to 100% oxygen up to 48 hours followed by a sharp decrease between 48 and 54 hours. A decrease in the specific activity of glutamine synthetase and glucose-6-phosphate dehydrogenase was observed under each experimental condition and preceded the loss of immunological cross-reactivity indicating the accumulation of inactive proteins. The level of acid proteases showed little variation under both conditions; however, the alkaline protease activity decreases with age and remains constant during the first 48 hours of oxygen exposure and then increases between 48 and 54 hours. Alkaline protease activity varied inversely with the accumulation of oxidized protein, suggesting that accumulation is due in part to a loss of proteolytic activities responsible for the degradation of damaged protein.

These results suggest that the accumulation of oxidized protein and the inactivation of enzymes which occur when animals are exposed to a high concentration of oxygen may reflect an acceleration of the same metabolic changes

that lead to the accumulation of oxidized proteins and enzyme inactivation during aging and accelerated aging diseases.

In view of the fact that diet restriction has been found to increase the life span of rodents, the effects of diet restriction on the level of oxidized liver proteins was determined. There was no significant difference in the level of carbonyl group in protein from rats fed ad libitum as compared to the levels in diet-restricted animals.

Since extensive exercise has been shown to affect the life span of rats, the carbonyl content of proteins in the hind leg muscle from groups of trained (exercised) rats and sedentary rats was determined. The carbonyl content of muscle protein from the trained animals was significantly greater than that from the sedentary animals. After two weeks of no exercise, the carbonyl content of the trained animals returned to the same level as found in the sedentary rats.

A sensitive assay has been developed to detect oxidatively modified proteins in human pulmonary lavage specimens. This assay can be used to detect oxidized proteins which may occur in certain diseases, or as a result of exposure to air pollutants.

(c) Regulation of Glycerol Metabolism in Klebsiella aerogenes. When Klebsiella aerogenes is switched from anaerobic to aerobic growth on glycerol, glycerol dehydrogenase undergoes rapid inactivation and degradation. The process requires synthesis of RNA and protein. It can also be triggered by exposure of the cells to hydrogen peroxide by a mechanism that is independent of protein synthesis.

(d) Amino Acid Oxidation. The Fenton reagent (hydrogen peroxide plus Fe(II) or Fe(III)) catalyzes nearly quantitative conversion of amino acids to ammonia and a mixture of aliphatic aldehydes and carboxylic acids containing one less carbon atom. The oxidation is stimulated by bicarbonate ion and additionally by a number of chelating agents when they are pesent at concentrations below that required to chelate all of the iron present. With excess chelator, amino acid oxidation occurs only after a lag which corresponds to the time required to reduce (by oxidation) the concentration of chelator to a level slightly below that needed to chelate all of the iron. The results suggest that amino acid oxidation requires the participation of both an iron-chelate and either unchelated iron or a second type of iron complex, possibly one involving bicarbonate ion and/or the amino acid. Low sensitivity of amino acid peroxidation to various radical scavengers indicates that oxygen radicals are either not involved or are generated in situ by peroxidation of an iron-chelate-amino acid complex. The formation of such a complex was established in spectrophotometric studies showing that Fe(II), ferrozine and an amino acid react to form first a ternary complex which subsequently decomposes to yield the typical (ferrozine)3-Fe(II) complex. The amino acid-Fe(II)-ferrozine-complex but not the (ferrozine)3-Fe(II) complex is readily oxidized by hydrogen peroxide.

(e) Oxidation of Homo Amino Acid Polymers. The oxidation of homopolymers of L-proline, L-histidine and L-lysine by Fenton's reagent leads to the fragmentation of the polypeptide chains and to the production of carbonyl groups. After acid hydrolysis, 5-oxo-2-amino pentanoic acid, cis-and trans-4-hydroxy proline were identified among the products of oxidized polyproline.

(f) Neutrophil Function. During the period of phorbol ester-induced respiratory burst, human neutrophils catalyze the covalent attachment of exogenously added isotopically-labeled tyrosine to a number of endogenous proteins. Dityrosine was identified among the products of acid hydrolysates of the endogenous proteins. However, the dityrosine was unlabeled and was therefore derived exclusively from endogenous tyrosine derivatives. The identity of the isotopically labeled tyrosine protein adduct is still unknown; it is alkali stable and acid labile.

Detection and Regulation of Phosphotyrosine Modification in Cellular Proteins

The phosphorylation and dephosphorylation of tyrosine residues on proteins has been implicated in the regulation of receptor function and in retrovirus gene transformation. Studies have therefore been carried out to develop sensitive techniques for the assay of protein tyrosine kinase and protein phosphotyrosine phosphatase activities, and for the detection of phosphotyrosine residues in proteins.

(a) Protein Phosphotyrosine Phosphatase. Calcineurin, a putitative protein phosphatase of broad specificity was found to catalyze the dephosphorylation of glutamine synthetase phosphotyrosine at only 1/1000 the rate of hydrolysis of nitrophenyl phosphate. This suggests that calcineurin resembles other nonspecific phosphomonoesterases and is not primarily concerned with the dephosphorylation of tyrosine-P residues of proteins.

Orthovanadate, an inhibitor of some PTP phosphatases was found to increase the levels of protein tyrosine phosphate *in vivo*.

(b) Detection of Protein Phosphotyrosine. Antibodies to tyrosine-P and specific antiprotein antibodies have been used inconjunction with SDS PAGE affinity chromatography and Western blot techniques to demonstrate the presence of phosphotyrosine residues in particular proteins. With these techniques, phosphotyrosinecalpactin was shown to be present in extracts of eye lens, and the concentration of protein phosphotyrosine residues was enhanced when 3T3-L1 adipocytes were treated with insulin, insulin-like growth factors 1 and 2, epidermal growth factor, and platelet-derived growth factor.

Regulation of Glutamine Synthetase Levels in Escherichia coli

It was previously reported that addition of a group of D-amino acids and glycine to *Escherichia coli* during exponential growth in a mineral salts medium will override the ability of high concentrations of ammonium salts to repress the synthesis of glutamine synthetase (GS). In continuing studies, it was found that the amino nitrogen of the D-amino acids is used for the synthesis of L-serine presumably in response to an observed increase in the level of serine hydroxymethyltransferase activity (SHMT). D-amino acid has no effect on the synthesis of GS in strains containing mutations in *glyA*, the structural gene for SHMT. Moreover, in contrast to the parental strains, the synthesis of GS was not derepressed when the *glyA* strains were grown on an ammonium nitrogen deficient medium. From further studies it was deduced that the ability of D-amino acid and glycine to facilitate derepression of GS synthesis in the presence of ammonium salts invovles three different genes: namely: genes coding for serine-hydroxymethyltransferase, phosphoribosyl-formylglycineamidine synthetase, and the $P_{II}$ regulatory protein of the GS cascade.

Annual Report of the
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The research projects of the investigators in the Section on Metabolic Regulation are mainly concerned with the physical, chemical, and biological approaches to resolve the mechanisms of enzyme action and its regulation. In the past year, research has been concentrated on (1) investigating the mechanisms of enzyme action and regulation which includes the phosphotidylinositol-specific phospholipase C, Ca(II)-calmodulin-dependent protein phosphatase and α-glycerol-3-phosphate dehydrogenase; (2) theoretical and experimental studies of energy and signal transduction via an electroconformational coupling mechanism, and (3) developing the mathematical basis to explain the anomalous stoichiometry of protein-ligand and protein-protein interactions using the continuous variation method, and improving the method for calculating King-Altman patterns. These research programs will provide a better understanding of how enzymes in living cells are regulated and how they work, and improve the methodology for biochemical research.

## I. Mechanism of Enzyme Action and Regulation

### A. Phosphotidylinositol-specific Phospholipase C

(1) Purification and characterization of phospholipase C isozymes. Phosphotidylinositol-specific phospholipase C plays an important role in initiating signal transduction through cell surface receptor, by generating two second messenger molecules, diacylglycerol and inositol-1,4,5-triphosphate. It has been shown by other workers that phospholipase C activity in mammalian brain can be resolved in several fractions. We, for the first time, have purified three phospholipase C isozymes (PLC-I, II, and III) from bovine brain to homogeneity. The purification procedure includes ion exchange chromatography on DE52, heparin-agarose chromatography, reverse phase chromatography on TSK phenyl 5PW and ion exchange chromatography on MonoS column. The apparent molecular weights of the three isozymes determined under denaturing conditions are 150,000, 145,000, and 85,000 for PLC-I, II, and III, respectively. PLC-I and III exist primarily as dimers, while PLC-II is present as a monomer. The catalytic properties of the three isozymes were investigated using small unilamellar vesicles prepared from either phosphatidylinositol or phosphotidylinositol-4,5-bisphosphate as substrate. The pH optima for PLC-I, II, and III are 4.8, 5.0, and 5.5, respectively. The specific activities are similar at their optimal pH; however, at neutral pH, the specific activity of PLC-III is higher than PLC-II whose specific activity is higher than PLC-I. With phosphotidylinositol as substrate, the hydrolysis rates catalyzed by PLC-II and PLC-III increase with increasing Ca(II) concentration up to 10 mM, while the activity of PLC-I only increased moderately and reaches a maximum at 6 mM. Although the specific activity for hydrolyzing this substrate for the three isozymes is similarly low when Ca(II) concentration is below 10 M, at high Ca(II) concentration they are significantly different. For example, the specific activity was found to be 2, 8 and 24 mol/min/mg for isozymes I, II, and III, respectively at 10 mM Ca(II). With phosphatidylinositol-4,5-bisphosphate as substrate,

the concentration of Ca(II) required to obtain maximum activity for the three isozymes is significantly lower than that required for phosphotidylinositol as substrate. At all Ca(II) concentrations studied, the relative activity found is PLC-I > PLC-III > PLC-II. In addition, phosphatidylinositol-4,5-bisphosphate is a much better substrate than phosphatidylinositol for PLC-I and PLC-III catalyzed hydrolysis, while PLC-II is less discriminating between the two substrates.

The divalent metal ion effect on the activities of PLC-I and PLC-II was also studied. The data show that in the micromolar concentration range, Hg(II), Cu(II) and Zn(II) inhibit both the activity of PLC-I and PLC-II, and that PLC-II is about 5-fold more susceptible to these metal ions than PLC-I. Cd(II) inhibits only PLC-II and not PLC-I. The inhibition caused by Hg(II) can be reversed by DTT but not EDTA, while EDTA but not DTT reverses the inhibition caused by Zn(II) and Cd(II). In the case of Cu(II)-induced inhibition, either DTT or EDTA can partially reverse it. Other divalent metal ions such as Co(II), Mn(II), Ni(II), and Mg(II) exhibit no effect on the activity of both isozymes.

(2) Immunological characterization of phospholipase C isozymes. Murine monoclonal antibodies directed against PLC-I and PLC-II were prepared. The competition experiments showed that anti-PLC-I antibodies, K-32-3, K-82-3, and K-92-3, recognize different epitopes on PLC-I and that anti-PLC-II antibodies, A-2-5, B-12-5, E-8-4, B-16-5, D-7-3, and E-9-4 bind to different sites on PLC-II. Specificity of these antibodies to either PLC-I or PLC-II was determined by binding experiments with immunoaffinity gels. Each of the three immunoaffinity gels prepared with the anti-PLC-I antibodies retained > 95% of PLC-I acitivity while less than 5% of PLC-II activity was retained. The immunoaffinity gels prepared with the six anti-PLC-II antibodies retained PLC-II ( 100%) but not PLC-I. Immunoblots of the SDS-PAGE gel were carried out by using individual monoclonal antibodies as well as their mixtures. Each of the three anti-PLC-I antibodies, K-32-3, K-82-3, and K-92-3, independently recognized 150 KDa PLC-I polypeptide but not 145 KDA PLC-II. Similarily, each of the six anti-PLC-II antibodies, A-2-5, B-12-5, E-8-4, B-16-5, D-7-3, and E-9-4, was specific to 145 KDa PLC-II polypeptide. Monoclonal antibodies against PLC-III is not yet characterized. However, studies with anti-PLC-III polyclonal antibody indicates that PLC-III is distinct from PLC-I and PLC-II.

(3) Distribution of the phospholipase C isozymes. Subcellular distribution of PLC-I and PLC-II in bovine brain homogenates was measured using radioimmunoassay. It was found that more than 90% of PLC-II is associated with the cytosolic fractions, whereas the PLC-I-like molecules are equally distributed between the cytosolic and particulate fractions. Purification of PLC enzyme to homogeneity from the particulate fractions yielded two PLC enzyme fractions, one with a molecular weight of 150,000 and the other 140,000. Both polypeptides are recognized by anti-PLC-I antibodies but not by anti-PLC-II antibodies. The 150 KDa enzyme is the same as PLC-I based on trypsin-digested peptides elution profile and molecular weight data. Therefore, we proposed that PLC-I and its truncated form are weakly associated with membranes.

Radioimmunoassay was also used to quantitate PLC-I and PLC-II in various rat organs and in cultured cells. The data show that more than 95% of PLC-I found is localized in the brain tissue while PLC-II is widely distributed. In addition, PLC-I also was found mainly in brain cultured cells while significant amounts of PLC-II was found in all cultured cells examined.

Immunohistochemical analysis of rat brain showed that both PLC-I and PLC-II were distributed equally in the cortex where they were found in neurons, in most neocortical areas and in the hippocampal pyramidal and dentate gyrus granule cells. The densest labeling of both PLC isozymes is in the basal ganglia, in the medium spiny projection neurons of the striatum. However, PLC-II but not PLC-I was found in both globus pallidus and substantia nigra. Both PLC-I and PLC-II are expressed in the thalamus, and PLC-I appears to be restricted to the thalamic reticular nucleus and to the mediodorsal thalamic nucleus, while PLC-II is more broadly distributed. In particular, neurons in the medial habenula express PLC-II but not PLC-I, consistent with PLC-II being found in the terminal fields of interpeduncleur nucleus. In the hypothalmus, PLC-I is absent in the paraventricular nucleus and PLC-II is present in a subset of parvocellular neurons.

B. On the Metabolite Transfer via Enzyme-Enzyme Complexes of Glycolytic Pathway

S. A. Bernhard recently proposed that metabolites in the glycolytic pathways may proceed by direct transfer from one enzyme site to the next by means of enzyme-enzyme complex formation (Spivastown and Bernhard, Curr. Top. Cell. Regul. 28, 1-28, 1986; Science 234, 1081-1086, 1986). The proposed mechanism differs from the normally assumed mechanism where metabolite is transferred from one enzyme site to the next enzyme site by means of dissociation and random diffusion through the aqueous environment. Among the experimental evidence reported in support of the proposed mechanism are the following: (1) The $K_m$ for dihydroxyacetone phosphate in α-glycerol-3-phosphate dehydrogenase catalyzed reaction is 220 μM. However, with the added micromolar concentration range of aldolase, the $K_m$ was reduced to 8 μM. The reduction in $K_m$ was attributed to the formation of aldolase-dihydroxyacetone phosphate complex (with $K_d \sim 1$ μM) which will then form a complex with α-glycerol-3-phosphate dehydrogenase, thus allowing the transfer of the aldolase bound dihydroxyacetone phosphate to the active site of α-glycerol-3-phosphate dehydrogenase. (2) Transient kinetic evidence suggests that NADH (reduced nicotinamide adenine dinucleotide) is transferred directly from one dehydrogenase enzyme site to another without going through the aqueous medium whenever the two dehydrogenases are of opposite chiral specificity with respect to the $C_4$ hydrogen of NADH. In addition, the rate coefficients for the transfer of NADH from one form of dehydrogenase to the other are relatively constant ($\sim$ 142-232 $sec^{-1}$), particularly in the cases of α-glycerol-3-phosphate dehydrogenase where $k_{off}$ is reported to be 9 $sec^{-1}$, while $k_{off}$ for glyceraldehyde-3-phosphate dehydrogenase is larger than 350 $sec^{-1}$. Yet their $K_{transfer}$ of NADH from each of these enzymes to lactate dehydrogenase is almost equal (142 $sec^{-1}$ for α-glycerol-3-phosphate dehydrogenase and 232 for glyceraldehyde-3-phosphate dehydrogenase). In other words, the dissociation rate of NADH from α-glycerol-3-phosphate dehydrogenase in the presence of lactate dehydrogenase was enhanced significantly while the $k_{off}$ from glyceraldehyde-3-phosphate dehydrogenase in the presence of lactate dehydrogenase was suppressed.

In view of the fundamental importance of the proposed mechanism and the fact that there is lack of rigor in data treatment, we decided to carry out some of the key experiments which are reported to be supportive for the proposed mechanism. The preliminary results of our data show: (1) Contrary to reported data, we found that aldolase is an inhibitor for α-glycerol-3-phos-

phate dehydrogenase catalyzed reduction of dihydroxyacetone phosphate. The inhibition is derived from aldolase binding to the substrate, dihydroxyacetone phosphate, and the $K_i$ obtained is about 30 μM which is in agreement with the reported $K_d$ for aldolase-dihydroxyacetone phosphate complex. (2) Transient kinetic studies revealed that the reported first-ordered rate for the transfer of NADH from α-glycerol-3-phosphate dehydrogenase to lactate dehydrogenase is a composite of two reactions -- one due to free NADH binding to lactate dehydrogenase and the other due to complex formation between NADH bound α-glycerol-3-phosphate and lactate dehydrogenase. (3) The enzyme bound NADH can be transferred directly to lactate dehydrogenase to serve as its substrate, and the rate constant for this transfer process is around 10 $sec^{-1}$. (4) The $k_{off}$ of NADH from α-glycerol-3-phosphate dehydrogenase is significantly larger than the reported 10 $sec^{-1}$ which was determined by displacement experiment using 10 mM NAD. The observed $k_{off}$ of the displacement reaction is NAD concentration dependent when the concentration was varied from 10 mM to 40 mM. So far, our results are not in agreement with those reported earlier by Srisvastava and Bernhard. However, there is evidence in support of complex formation, and the enzyme-enzyme complex is functionally active.

C. Mechanistic and Structural Studies on Calmodulin-dependent Protein Phosphatase. The calmodulin (CaM)-dependent protein phosphatase (CPP) is a Zn(II)- and Fe(II)-containing enzyme which requires an additional divalent metal ion such as Ni(II), Mn(II), Co(II), Mg(II), etc. for structural stability and catalytic activity. CPP, as isolated, exists in both phosphorylated and dephosphorylated forms, though the functional significance of this covalent modification is not yet understood. In the absence of CaM, CPP is activatable by Ni(II) provided some Ca(II) is present. The time course of Ni(II) activation exhibits an initial lag phase which, unlike that observed in the presence of CaM, does not follow first-order reaction kinetics. When lag times were plotted against reciprocal Ni(II) concentrations, a nonlinear (concave downward) relationship was evident. Thus, the activation mechanism was proposed by us to involve the random binding of two Ni(II) ions. This mechanism is different from that proposed by Pallen and Wang (J. Biol. Chem. 259, 6134, 1984) in which binding of one Ni(II) induced the enzyme to two active forms. Reexamination of the experimental evidence in support of Pallen and Wang's mechanism revealed that their conclusion was derived from an artifact due to pH shift caused by EDTA binding to Ni(II). In addition, theoretical consideration shows that the later mechanism will not explain the kinetic data observed.

$^{63}$Ni(II) binding data obtained in either the presence or absence of CaM are similar even though CaM plays a role in Ni(II) activation. The amount of bound Ni(II) was about 0.8 mole per mole of enzyme after 2 minutes incubation and about 1.8 mole per mole of enzyme after 60 minutes. The results support the concept that two mole of Ni(II) per mole of CPP are irreversibly bound. Furthermore, our work on phosphorylation of CPP by protein kinase C has been extended to show that freshly prepared CPP can also be phosphorylated by the catalytic fragment of protein kinase C and c-AMP-dependent protein kinase. However, CPP stored at -70°C for several months could not be phosphorylated by either kinase.

D. Purification and characterization of Ca(II)-sensitive Phosphatase from Bovine Brain

Using PNPP as substrate, we have purified a Ca(II)-inhibited phosphatase to >80% purity from bovine brain. The procedure includes homogenation and centrifugation, $(NH_4)_2SO_4$ cuts, DEAE-cellulose chromatography, S-200 gel filtration, CaM-sepharose column, and Mono-Q column (FPLC system). Gradient gel electrophoresis of the final sample revealed one major active band of $M_r \sim 56,000$ and two minor bands. However, when phosphorylated histone 2B was used as substrate, we found several Ca(II)-sensitive phosphatases whose molecular weights range from 20,000 to 200,000 in the protein fractions from DEAE-cellulose and S-200 column steps. The 200,000 $M_r$ phosphatase does not hydrolyze PNPP.

E. On the Purification and Characterization of Isopeptidase

Ubiquitin, a 76-residue ($M_r$ = 8565) heat-stable protein is known to conjugate to proteins via the ε-amino or the α-amino groups. It has been proposed that protein ubiquitination may play a role in DNA transcription (ubiquitination of histone $H_{2A}$) and in ATP-dependent protein turnover in eukaryotes. Isopeptidase catalyzes the deubiquitination reaction. Thus, together with the enzyme system catalyzing the ubiquitination reaction, isopeptidase consitutres a new cyclic cascade involving ubiquitination/deubiquitination of proteins. To purify isopeptidase, we first constructed a suitable assay system. This involved a chemical synthesis of a ubiquitin lysozyme conjugate. The procedure used included modification of the C-terminus of ubiquitin to an ethyl ester, then converting it to its azide derivative and finally the azide was displaced by the primary amines of lysozyme. Although the synthetic procedure is relatively straightforward, a complication was found due to, in part, the ubiquitin conjugates lysozyme aggregate easily.

II. Effect of Oscillating Membrane Potentials on the Dynamics of Biological Signal and Energy Transduction

A. Theoretical Treatment

Transmembrane potential plays an important role in determining the activity of membrane bound proteins. Although the amplitude of a typically observed oscillating transmembrane potential is modest, e.g, 50 mV range, for living cells, it represents an electric field strength of 100,000 V/cm for a 50 Å membrane. This magnitude of field strength is large enough to induce shift in conformational equilibria of many membrane proteins. Therefore, the physiologically attainable oscillation in transmembrane potential is capable of regulating the function of many membrane-bound enzymes. It is believed that biological systems probably make use of these macroscopic oscillations for energy and signal transduction. A theory has been formulated to explain how oscillating or fluctuating membrane potential can be utilized to modulate the activities of enzymes involved in transducing biological signal and energy. In developing the theory, we took into consideration the fact that the conformational transitions of many transmembrane proteins have a significant electric susceptibility such that their conformational states and thus their enzymic activities can be modulated through changes in electric field. This is particularly evident in the case of voltage gated channels and in cases where

protein conformational change involved either intramolecular charge transfer or rotation of dipole groups such as α-helices. The results of the theoretical calculation revealed that energy can be transduced from oscillating or fluctuating fields and the efficiency and efficacy for such energy transduction is comparable to experimentally measurable ones. The proposed theory provides a very general mechanistic formulation through which energy transduction via macromolecules can be understood. The extension of this theory can also provide a mechanistic explanation on how cytosolic free energy transduction based solely on protein-protein interactions works.

B. Experimental Approach

Experimental work designed to verify the validity of the above theory is planned. For this purpose, we are developing an apparatus which can be used to apply an oscillating electric field across a suspension of cell with a variable amplitude up to ± 150 V/cm and a variable wave form. At present, the apparatus can monitor the transient conductance of both sample and reference cell and the data are stored in a computer for subsequent analysis. By comparing the Fourier spectrum of the input periodic potential and the output current signal, one can detect and analyze the nonlinear responses of membrane processes to stimulation. We hope this will allow one to evaluate rate constants for the electric field-induced membrane processes.

## III. Analytical Methods

A. Anomalous Stoichiometry of Protein-Ligand and Protein-Protein Interactions As Determined by the Continuous Variation Method (Job Plot).

In the continuous variation method, the total molar conclentration, $C_o$, of two interacting components is held constalnt while thier mole fractions, X and Y are continuously varied. A measurable parameter that is linearly proportional to the complex(es) formed, Σ, is plotted against the mole fractions, and the intersection point of the two limiting slopes, $d\Sigma/dX)_{X\to o}$ and $d\Sigma/dY)_{Y\to o}$, are determined. The binding stoichiometry, n, is calculated from the ratio of the mole fractions of these two components at the intersection point of these slopes, $Y_i/X_i$. Normally, the observed n value varies between one and n as $C_o$ increases relative to the magnitude of the dissociation constant(s) involved. Recent developments indicate that under certain circumstances the apparent stoichiometry can become greater than the true n value. These anomalous situations have been shown theoretically to exist in binding studies involving cooperativity or reconsitituion of enzyme with unidentical subunit. Therefore, it is necessary to perform the Job plot experiments with a wide range of $C_o$ in order to obtain the correct n value and to gain knowledge of the mode of binding.

B. Improved Graphical Method for Calculating King-Altman Patterns for Complex Kinetic Reactions.

The graphical method for calculating King-Altman patterns, originally developed by Huang, permits rapid computation of the total valid patterns in most instances. An improved procedure has been devised for highly complex reactions which may involve three-dimensional schemes and for calculation of the exact number of patterns of individual enzyme species in a scheme containing

irreversible steps. Basically, the method involves breaking the parent scheme into two diagrams by selecting a reference branch such that in one diagram the reference branch is deleted, while in the other it is obligatory. In the latter diagrm, the selected branch is compressed into a point. This subdiagramming procedure can be repeated as many times as necessary until the resultant diagrmas can be easily calculated. The improved method has been shown to work effectively with three-dimensional reaction schemes even when the scheme is complex and contains two subschemes linked by a common node, and to reaction schemes which contain irreversible steps.

C. Determination of Divalent Metal Ion Binding Constants for Ca(II) Indicators

To study the role of calcium as an intracellular messenger requires monitoring the concentration of cytosolic calcium. A family of Ca(II) indicators which contain ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid (EGTA) backbone as a common denominator has been synthesized by R. Y. Tsien and coworkers. These indicators are highly fluorescent and they bind Ca(II) tightly, therefore they are excellent Ca(II) indicators. In addition, the ester derivatives of these indicators are sufficiently nonpolar such that they can permeabilize across the plasma membrane and be hydrolyzed intracellularly to return to their membrane-impermeable polycarboxylate anions. In order to properly quantitate the free Ca(II) concentration using these indicators, it is necessary to determine accurately the binding constants of these indicators for Ca(II) and other metal ions which may interfere with Ca(II) binding. Since these indicators, Indo 1 (see figure), Fura 2, and Quin 2 bind most divalent metal ions very tightly such that the direct titration method is not suitable for determining binding constants, we used both the kinetic method and the competition titration method to evaluate the binding constants. The metal ion studied are Ca(II), Ni(II), Fe(II), Fe(III), Mg(II), Mn(II), Zn(II), Co(II), Cd(II), and Cu(II).

Annual Report
Section on Intermediary Metabolism
and Bioenergetics
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1986 to September 30, 1987

It now is well documented that selenium is an essential trace element for animals, several types of bacteria, and also a few photosynthetic organisms. Our studies on the biochemical roles of selenium at the enzyme level have dealt with certain selenium-dependent enzymes that occur in bacteria. We also have identified selenium modified amino acid transfer ribonucleic acids (seleno-tRNAs) and investigated their mode of biosynthesis and metabolic roles.

Selenium is present in clostridial glycine reductase in the chemical form of a single selenocysteine residue located in the 12 kDa protein A component of the reductase complex. Oligonucleotide probes complimentary to the amino acid sequence around the selenocysteine residue were synthesized and used to select DNA fragments from a Clostridium sticklandii gene library. Because of considerable redundancy the clones so far selected from the DNA library have not proven to be the selenoprotein A gene. A new synthetic oligonucleotide probe that includes UGA, the presumed selenocysteine codon, will be used for screening. Also, a new gene library, enriched for sequences that hybridize to the 20 mer oligonucleotide probe already available, will be created. When found, the selenoprotein A gene will be sequenced to determine if the stop codon, TGA, is used by the clostridium to specify selenocysteine incorporation. This gene should be especially useful for transcription-translation studies on mechanism of selenocysteine incorporation into proteins. Also, if over expressed, larger amounts of the selenoprotein A will be useful for studies on the mechanism of the glycine reductase reaction.

In a collaborative research project with August Böck (University of München), it was shown that the gene encoding a formate dehydrogenase of Escherichia coli contains the termination codon, TGA, in the open reading frame. Read through of the TGA (UGA) codon when a portion of the formate dehydrogenase gene is fused to the β-galactosidase gene was shown to require the addition of selenium in the medium. When the TGA codon was changed to TGC or TGU (cysteine codon), selenium was no longer required for synthesis of the fusion protein. A variety of indirect genetic pieces of evidence indicate that selenocysteine is inserted as a co-translational event in response to the UGA stop codon. Our experimental approach to the problem is to devise an in vitro transcription-translation system using various gene constructs and more efficient promoters in order to identify the tRNA and protein factors that allow usage of the stop codon for selenocysteine incorporation into protein. So far it appears that the selenation part of the system may be the rate limiting part of the process. In attempts to correct this deficiency additional protein fractions and enriched tRNA preparations from clostridia and methane bacteria will added to the E. coli system.



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A procedure that appears to be suitable for isolation of the unstable selenocysteine peptide from the E. coli formate dehydrogenase has finally been devised. This is needed for amino acid sequence determination around the selenocysteine residue to prove that the TGA codon in the DNA sequence matches the selenocysteine residue location in the protein product.

Studies on the in vivo expression of the E. coli formate dehydrogenase-β-galactosidase fusion gene show that expression is considerably increased when DNA supercoiling is decreased. In these studies DNA supercoiling was decreased by addition of chemical inhibitors, e.g., coumermycin, to inhibit DNA gyrase activity or by use of temperature-sensitive mutants. The observed increase in expression of the strictly anaerobic formate dehydrogenase concomitant with inhibition of DNA gyrase is in direct contrast to results found for a few other anaerobically controlled genes which appear to require supercoiled DNA. Presumably, changes in geometry of promoter regions due to decreased or increased supercoiling of the DNA can account for these effects. As an experimental test system, E. coli strains containing deletions in various locations of the formate dehydrogenase promoter region will be examined.

In previous studies on seleno-tRNAs in various bacterial species, two selenonucleosides were isolated and identified. These identifications were made possible by availability of authentic synthetic selenonucleosides prepared in our laboratory. These compounds are 5-methylaminomethyl-2-selenouridine and 5-aminomethyl-2-selenouridine. A third conspicuous selenonucleoside isolated from the tRNA population of Methanococcus vannielii was thought to have a $-CH_2NHCH_2COOH$ side chain in the five position of the 2-selenouridine residue. A synthetic procedure for preparation of this selenonucleoside and its 2-thio analogue has been developed. Recent reports that the $-CH_2NHCH_2COOH$ side chain is a precursor of the $-CH_2NH_2$ and the $-CH_2NHCH_3$ side chains make this an interesting substrate for biochemical studies.

As part of the program dealing with the role of trace elements in metabolism, studies were made on enzymes that contain nickel and molybdenum as essential components. Carbon monoxide dehydrogenase, a nickel-iron sulfide enzyme, is involved in the anaerobic carbon dioxide fixation pathway that forms acetate by the direct condensation of $C_1$ units. In the reverse direction, the anaerobic cleavage of acetate to methane and carbon dioxide, carbon monoxide dehydrogenase also appears to play an essential role. The homogeneous enzyme isolated from Methanosarcina barkeri that grows on acetate and produces $CH_4$ and $CO_2$ proved to be identical with the enzyme isolated in pure form from Methanococcus vannielii that grows on formate as sole carbon source and synthesizes acetate from $C_1$ units as a building block for cell material. The subunit structure, extreme oxygen sensitivity, and metal composition of the enzymes from the two sources are almost identical. These enzymes differ from a clostridial carbon monoxide dehydrogenase that synthesizes acetyl-CoA from a methyl group and carbon monoxide. Sensitivity of the enzymes from the methane bacteria to various inhibitors was determined as a basis of studies on reaction mechanism.

Another enzyme, xanthine dehydrogenase, also was isolated in pure form from Methanococcus vannielii. This enzyme functions in the purine degradation pathway which allows the methane organism to use purines as nitrogen source

for growth. The xanthine dehydrogenase contains molybdenum in the form of a molybdo-pterin cofactor and also numerous iron-sulfur centers. The subunit structure and some of the properties of the pure enzyme were determined.

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Annual Report of the
Laboratory of Biochemistry
Section on Protein Chemistry
National Heart, Lung, and Blood Institute
October 1, 1986 to September 30, 1987

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The Section on Protein Chemistry is studying the physical and chemical properties of macromolecules of biological interest and 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 involve 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 many examples of stabilization and destabilization of protein structures by ligands and metal ions.

Glutamine synthetase, a strictly regulated enzyme in Escherichia coli, is a dodecamer; each subunit (51,814 $M_r$) 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 the enzyme are arranged in 2 superimposed hexagonal rings ~ 140 Å in diameter and centers of adjacent subunits are ~ 45 Å apart. Interactions of divalent cations, substrates, substrate analogues, and inhibitors with glutamine synthetase from E. coli have been studied.

The very tight binding of 2 $Mn^{2+}$, L-methionine-S-sulfoximine-phosphate, and ADP ($K_A' > 10^{12}\ M^{-1}$) to each subunit of E. coli glutamine synthetase at pH 7 after phosphorylation of the L-glutamate analogue by ATP stabilizes inter-subunit bonding domains. Various analogues of ATP that are substituted at the 6- or 8-position of the adenine ring are 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-site nucleotide probes of the enzyme has been measured by fluorescence energy transfer, taking advantage of the essentially irreversible binding of various ADP analogues at neutral pH when bound with L-met-S-sulfoximine-phosphate and $Mn^{2+}$ at active sites. Last year we reported that active-site nucleotide probes on the dodecamer are widely separated (> 56 Å) and that energy transfer occurred from a single donor to 2 or 3 acceptors on adjacent subunits. The results obtained in this laboratory are entirely consistent with the atomic model for glutamine synthetase recently proposed by D. Eisenberg's group at UCLA from X-ray crystallographic analysis (Almassy et al, Nature 323, 304-309, 1986). An unusual feature of the enzyme structure is that the 12 active sites are formed at heterologous interfaces between subunits within a hexagonal ring of the two face-to-face eclipsed rings. The two $Mn^{2+}$ at an active site are in the C-terminal domain, whereas the nucleotide binding site is in the N-terminal region (near Lys 47) of an adjacent subunit. This explains how the binding of substrates stabilizes inter-subunit contacts of the enzyme. The distance between $Mn^{2+}$ ions at neighboring active sites within the same ring of six subunits is ~ 45 Å (Almassy et al.) which is less than that estimated between nucleotide probes by fluoresence energy transfer measurements. Apparently, the adenosine moiety of an ATP molecule is more towards the exterior surface of the enzyme than are bound $Mn^{2+}$ ions. In attempts to solve this and other questions

prompted by the proposed atomic model, we have introduced an electron dense nucleotide•Pt(II) complex at either active sites or adenylylation sites and these enzyme derivatives have been sent to D. Eisenberg for crystallization and X-ray analysis. Interestingly, several highly regulated enzymes have been shown to have active sites formed across inter-subunit contacts of oligomeric proteins. Possibly, this provides the necessary evolutionary pressure to select for regulation as well as for catalytic activity of key metabolic enzymes which require oligomeric structures for modulation of activity expression.

The stacking of glutamine synthetase dodecamers ($M_r$ 622,000) is being studied by calorimetry in order to investigate forces governing macromolecular assembly reactions. $Zn^{2+}$ binds to a site distinct from the active site of each subunit with $K_A' = 5 \times 10^6 M^{-1}$ at pH 7.0 and deforms the enzyme in such a way that when 50 mM $MgCl_2$ also is present, spontaneous face-to-face aggregation of enzyme dodecamers occurs. The rate of $Zn^{2+}$ induced polymerization of glutamine synthetase increases with increasing temperature with an Arrhenius activation energy of 17.7 kcal/mol of dodecamers--a small activation energy considering that $Zn^{2+}$ binding deforms the enzyme and that 6 intermolecular contacts must formed in the stacking process. Enthalpy changes ($\Delta H$) for (1) the binding of $Zn^{2+}$ to the enzyme and protein conformational changes, and (2) the $Zn^{2+}$-induced aggregation of the enzyme were measured by calorimetry at pH 7.0 and 22.5, 30.0, and 38.0°C. In the absence of $Mg^{2+}$, the addition of 0.7 eq of $Zn^{2+}$ per subunit produces no aggregation of the enzyme and $\Delta H = + 115$ kcal/mol of dodecamer for (1) at 38°C with $\delta\Delta H/\delta T = 1500$ cal/K•mol for $Zn^{2+}$ binding. Subtracting the latter value from that obtained for (1) + (2) gave $\Delta Cp \approx -1000$ cal/K•mol for protein-protein interactions in (2). The large $\Delta Cp$ value for (2) implicates a dominant role of water in the stacking process. Accordingly, pressure effects on the stacking of GS dodecamers currently are being investigated.

The thermodynamic parameters for active-site ligand binding to E. coli glutamine synthetase contain contributions from both protein-ligand and protein-protein interactions. The positive entropy change for interaction of the enzyme with a full complement of substrates could result from forcing the nucleotide into a more hydrophobic environment for interaction with the $\gamma$-carboxylate group of L-glutamate and concomitant changes in hydration. However, the ionic interactions of the $\gamma$-phosphate of ATP play a major role in contributing to the entropy changes observed. Also, interactions at the L-glutamate and ammonia sites produce ordering effects that are opposed by large exothermic heats of binding. Active-site ligand stabilization of intra- and inter-subunit bonding domains to produce a more compact dodecameric structure would be expected to lead to negative values of $\Delta H$ and $\Delta S$ through the strengthening of hydrogen bonds in the protein interior and van der Waals' interactions brought about by the partial withdrawal of nonpolar groups from water. These more remote stabilizing effects of active-site ligand binding appear to be offset by local charge neutralization effects at active sites.

Active-site ligand and metal ion interactions with mammalian octameric glutamine synthetase from bovine brain have been studied. The evidence obtained from binding and kinetic studies indicates 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 com-

plex ($K_A' \approx 10^6\ M^{-1}$) after the first site ($K_A' \approx 1.5 \times 10^5\ M^{-1}$) 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 ($K_A' \approx 10^4\ M^{-1}$) has been found to destabilize this enzyme and to promote both fluorescence and UV absorbance changes. The effects of chloride ions and those produced by an allosteric binding of L-glutamate 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 (ATCase; $c_6r_6$) and $Zn^{2+}$ uptake by regulatory dimers ($r_2$) upon displacement of the mercurial reagent with 2-mercaptoethanol. As previously reported, the properties of PAR-$Zn^{2+}$ interactions make PAR a generally useful reagent for studying $Zn^{2+}$ release from proteins. For example, stopped-flow measurements in the presence of 100 μM PAR at 500 nm of mercurial-promoted release of $Zn^{2+}$ from regulatory subunits (with $Zn^{2+}$ tetrahedrally bound by the 4 thiol groups of each r chain) was first-order with a half-time of 50 ± 3 ms at 15°C. This rate is ≥ 2800-fold faster than the corresponding rate of $Zn^{2+}$ release from intact ATCase in which $Zn^{2+}$ ions are bound near regulatory:catalytic chain (r:c) contacts. However, using $(PAR)_2Zn$ as the donor of $Zn^{2+}$ to a protein acceptor involves complex equilibria and possibly a rate-limiting release of $Zn^{2+}$ from PAR which must be coupled to the binding of $Zn^{2+}$ to the acceptor. Studies are in progress to determine the on-rate of $Zn^{2+}$ binding to $r_2$ subunit in the absence and presence of PAR. The results will show whether or not the PAR-$Zn^{2+}$ complexes can be used to obtain kinetic constants for $Zn^{2+}$ binding to a high affinity protein acceptor with $K_A' \approx 10^{13}\ M^{-1}$.

Possible roles of $Mn^{2+}$ and $Zn^{2+}$ in maintaining the quaternary structures r f arginase from yeast and bovine liver are being investigated. Arginase from yeast is a trimer, whereas the enzyme from liver reportedly is a tetramer. We have detected two active forms of arginase purified from bovine liver and with a newly acquired anti-arginase antibody produced by immunizing a rabbit with the purified liver enzyme, we can see if multiple active arginase forms arise from proteolysis. Also, we can use this antibody to check for cross-reactivity between yeast and liver arginases and to quantitate $Zn^{2+}$ and $Mn^{2+}$ in arginase from freshly broken cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00202-16 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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)

| | | | |
|---|---|---|---|
| PI: | P. Boon Chock | Chief, Section on Metabolic Regulation | LB, NHLBI |
| Others: | R. Dean Astumian | Staff Fellow | LB, NHLBI |
| | James Cook | Staff Fellow | LB, NHLBI |
| | Rixin Zhou | Volunteer | LB, NHLBI |

COOPERATING UNITS (if any)

T.Y. Tsong, Johns Hopkins U. Sch. of Med., Baltimore, MD; D. Yang, Georgetown U., Washington, D.C.; H. Gutfreund, Fogarty Scholar-in-Residence (Bristol U., Bristol, England)

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.75 | 2.95 | 0.85 |

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) Experiments designed to test the direct transfer mechanism for metabolites in the glycolytic pathway have revealed that the data used to support the proposed mechanism are questionable. Contrary to the report that aldolase forms a complex with α-glycerol-3-phosphate dehydrogenase and facilitates the dehydrogenase catalyzed reaction through direct transfer of aldolase-bound substrate, dihydroxyacetone phosphate, we found that aldolase inhibits the dehydrogenase-catalyzed reaction by removing substrate for α-glycerol-3-phosphate dehydrogenase. In addition, transient kinetic data show that the reported first-ordered transfer rate of NADH from α-glycerol-3-phosphate dehydrogenase to lactate dehydrogenase is a composite of two reactions, one due to free NADH binding to lactate dehydrogenase and the other due to complex formation between the two dehydrogenases.

(2) Binding constants for Ca(II) and a number of divalent metal ions have been determined for a family of Ca(II) indicators designed to probe intracellular calcium concentration.

(3) A theory has been developed to account for the effect of oscillating or fluctuating membrane potential on the function of membrane-bound proteins. The theory provides a reasonable mechanism for energy transduction. In addition, an oscillating electric pulse apparatus is being constructed to carry out experiments to test the validity of the theory.

(4) Work is in progress to purify and characterize isopeptidase.




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

Project Description

Objectives:

(1) A physical-chemical approach will be used to study the roles of enzyme-enzyme complexes in the direct transfer of metabolites in the glycolytic pathways.

(2) Theoretical analysis of the cyclic cascade system will be investigated to further reveal its properties and function in metabolic regulation of key enzymes.

(3) To purify and characterize isopeptidase and to investigate the roles of an ubiquitination/deubiquitination cascade.

(4) To conduct a theoretical and experimental investigation of the mode of energy and signal transduction.

(5) To investigate the role of calcium as a second messenger.

Major Findings:

(1) On the Metabolite Transfer via Enzyme-Enzyme Complexes of Glycolytic Pathway

S. A. Bernhard recently proposed that metabolites in the glycolytic pathways may proceed by direct transfer from one enzyme site to the next by means of enzyme-enzyme complex formation (Spivastown and Bernhard, Curr. Top. Cell. Regul. 28, 1-28, 1986; Science 234, 1081-1086, 1986). The proposed mechanism differs from the normally assumed mechanism where metabolite is transferred from one enzyme site to the next enzyme site by means of dissociation and random diffusion through the aqueous environment. Because the direct transfer mechanism does not involve the aqueous environment, the energetics of metabolite interconversion can differ from that predicted based on data obtained in aqueous solution. Among the experimental evidence reported in support of the proposed mechanism are the following: (1) The $K_m$ for dihydroxyacetone phosphate in α-glycerol-3-phosphate dehydrogenase catalyzed reaction is 220 μM. However, with the added micromolar concentration range of aldolase, the $K_m$ was reduced to 8 μM. The reduction in $K_m$ was attributed to the formation of aldolase-dihydroxyacetone phosphate complex (with $K_d \sim 1$ μM) which will then form a complex with α-glycerol-3-phosphate dehydrogenase, thus allowing the transfer of the aldolase bound dihydroxyacetone phosphate to the active site of α-glycerol-3-phosphate dehydrogenase. (2) Transient kinetic evidence suggests that NADH (reduced nicotinamide adenine dinucleotide) is transferred directly from one dehydrogenase enzyme site to another without going through the aqueous medium whenever the two dehydrogenases are of opposite chiral specificity with respect to the $C_4$ hydrogen of NADH. In addition, the rate coefficients for the transfer of NADH from one form of dehydrogenase to the other are relatively constant ($\sim$ 142-232 $sec^{-1}$), particularly in the cases of α-glycerol-3-phosphate dehydrogenases where $k_{off}$ is reported to be 9 $sec^{-1}$, while $k_{off}$ for glyceraldehyde-3-phosphate dehydrogenase is larger than 350 $sec^{-1}$. Yet their $K_{transfer}$ of NADH

from each of these enzymes to lactate dehydrogenase is almost equal (142 $sec^{-1}$ for α-glycerol-3-phosphate dehydrogenase and 232 for glyceraldehyde-3-phosphate dehydrogenase). In other words, the dissociation rate of NADH from α-glycerol-3-phosphate dehydrogenase in the presence of lactate dehydrogenase was enhanced significantly while the $k_{off}$ from glyceraldehyde-3-phosphate dehydrogenase in the presence of lactate dehydrogenase was suppressed.

In view of the fundamental importance of the proposed mechanism and the fact that there is lack of rigor in data treatment, we decided to carry out some of the key experiments which are reported to be supportive for the proposed mechanism. The preliminary results of our data show: (1) Contrary to reported data, we found that aldolase is an inhibitor for α-glycerol-3-phosphate dehydrogenase catalyzed reduction of dihydroxyacetone phosphate. The inhibition is derived from aldolase binding to the substrate, dihydroxyacetone phosphate, and the $K_i$ obtained is about 30 μM which is in agreement with the reported $K_d$ for aldolase-dihydroxyacetone phosphate complex. (2) Transient kinetic studies revealed that the reported first-ordered rate for the transfer of NADH from α-glycerol-3-phosphate dehydrogenase to lactate dehydrogenase is a composite of two reactions -- one due to free NADH binding to lactate dehydrogenase and the other due to complex formation between NADH bound α-glycerol-3-phosphate and lactate dehydrogenase. (3) The enzyme bound NADH can be transferred directly to lactate dehydrogenase to serve as its substrate, and the rate constant for this transfer process is around 10 $sec^{-1}$. (4) The $k_{off}$ of NADH from α-glycerol-3-phosphate dehydrogenase is significantly larger than the reported 10 $sec^{-1}$ which was determined by displacement experiment using 10 mM NAD. The observed $k_{off}$ of the displacement reaction is NAD concentration dependent when the concencentration was varied from 10 mM to 40 mM. Currently, we are carrying out experiments to confirm the complex formation between α-glycerol-3-phosphate dehydrogenase and lactate dehydrogenase. So far, our results are not in agreement with those reported earlier by Srisvastava and Bernhard. However, there is evidence in support of complex formation, and the enzyme-enzyme complex is functionally active.

(2) Determination of Divalent Metal Ion Binding Constants for Ca(II) Indicators.

To study the role of calcium as an intracellular messenger requires monitoring the concentration of cytosolic calcium. A family of Ca(II) indicators which contain ethylene-bis-(oxyethylenenitrilo)-tetraacetic acid (EGTA) backbone as a common denominator has been synthesized by R. Y. Tsien and coworkers. These indicators are highly fluorescent and they bind Ca(II) tightly, therefore they are excellent Ca(II) indicators. In addition, the ester derivatives of these indicators are sufficiently nonpolar such that they can permeabilize across the plasma membrane and be hydrolyzed intracellularly to return to their membrane-impermeable polycarboxylate anions. In order to properly quantitate the free Ca(II) concentration using these indicators, it is necessary to determine accurately the binding constants of these indicators for Ca(II) and other metal ions which may interfere with Ca(II) binding.

Since these indicators, Indo 1 (see figure), Fura 2, and Quin 2 bind most divalent metal ions very tightly such that the direct titration method is not suitable for determining binding constant, we used the kinetic method to evaluate the rate constants for both the association and dissociation processes for a given metal ion. This kinetically determined binding constant was then used to evaluate other binding constants through competition experiments. The divalent metal ion chosen for evaluating the kinetic constant is Ni(II) because its diffusion controlled association rate is reasonably slow and can be measrued using a stopped-flow instrument. The rate for the dissociation process was measured by displacing the Ni(II) with Ca(II). During this study, it was found that the carboxyl group of the indole moiety of Indo 1 (see figure) can provide a second metal ion binding site at millimolar concentration. It binds Ca(II) with an equilibrium dissociation constant of ~ 54 mM. When the second metal ion binding site is filled, it facilitates the off-rate of Ni(II) by seven-fold. The facilitation process can be due either to reduced dimensionality effect or to conlombic effect exerted by the indicator bound second metal ion. To differentiate these two effects, Sc(III) was also used to displace Ni(II) from the indicator. The enhancement factor with Sc(III) is 29 instead of 7. Therefore, it is concluded that the effect of the second bound metal ion on the off-rate of Ni(II) is purely conlombic. Using the kinetically determined binding constant for Ni(II), we measured the binding constants for other tightly bound metal ions by competitor-titration method. The constants so evaluated are given in Table I.

Table I. Dissociation constants in the unit of μM for the 1:1 complex of metal ion and indicator determined in 50 mM Hepes buffer (pH 7.0) containing 100 mM KCl at 20°C.

| Metal ion | Indo 1 | Fura 2 | Quin 2 |
|---|---|---|---|
| Ca(II) | 0.42 | 0.40 | 0.20 |
| Ni(II) | 0.058 | 0.18 | 0.0044 |
| Fe(II) | 0.045 | 0.16 | -- |
| Fe(III) | 1.7 | 3.5 | -- |
| Hg(II) | 0.44 | 0.38 | 0.0033 |
| Mn(II) | 0.012 | 0.011 | 0.00027 |
| Zn(II) | 0.0043 | 0.0047 | 0.0000084 |
| Co(II) | 0.0072 | 0.013 | 0.0026 |
| Cd(II) | 0.000026 | 0.000059 | 0.0000026 |
| Cu(II) | 0.000016 | 0.000027 | 0.00000022 |

(3) Effect of Oscillating Transmembrane Potential on the Function of Membrane Proteins (also see R. Dean Astumian's annual report).

Transmembrane potential plays an important role in determining the activity of membrane bound proteins. Although the amplitude of a typically observed oscillating transmembrane potential is modest, e.g, 50 mV range, for living cells, it represents an electric field strength of 100,000 V/cm for a 50 Å membrane. This magnitude of field strength is large enough to induce shift in conformational equilibria of many membrane proteins. Therefore, the physiologically attainable oscillation in transmembrane potential is capable of regulating the function of many membrane-bound enzymes. It is believed that biological systems probably make use of these macroscopic oscillations for energy and signal transduction. A theory has been formulated to explain how oscillating or fluctuating membrane potential can be utilized to modulate the activities of enzymes involved in transducing biological signal and energy. In developing the theory, we took into consideration the fact that the conformational transitions of many transmembrane proteins have a significant electric susceptibility such that their conformational states and thus their enzymic activities can be modulated through changes in electric field. This is particularly evident in the case of voltage gated channels and in cases where protein conformational change involved either intramolecular charge transfer or rotation of dipole groups such as α-helices. The results of the theoretical calculation revealed that energy can be transduced from oscillating or fluctuating fields and the efficiency and efficacy for such energy transduction is comparable to experimentally measurable ones. The proposed theory provides a very general mechanistic formulation through which energy transduction via macromolecules can be understood. The extension of this theory can also provide a mechanistic explanation on how cytosolic free energy transduction based solely on protein-protein interactions works.

Experimental work designed to verify the validity of the above theory is planned. For this purpose, we are developing an apparatus which can be used to apply an oscillating electric field across a suspension of cell with a variable amplitude up to ± 150 V/cm and a variable wave form. At present, the apparatus can monitor the transient conductance of both sample and reference cell and the data are stored in a computer for subsequent analysis. By comparing the Fourier spectrum of the input periodic potential and the output current signal, one can detect and analyze the nonlinear responses of membrane processes to stimulation. We hope this will allow one to evaluate rate constants for the electric field-induced membrane processes.

(4) Purification of Isopeptidase (also see James Cook's annual report).

Ubiquitin, a 76-residue ($M_r$ = 8565) heat-stable protein is known to conjugate to proteins via the ε-amino or the α-amino groups. It has been proposed that protein ubiquitination may play a role in DNA transcription (ubiquitination of histone $H_{2A}$) and in ATP-dependent protein turnover in eukaryotes. Isopeptidase catalyzes the deubiquitination reaction. Thus, together with the enzyme system catalyzing the ubiquitination reaction, isopeptidase consitutres a new cyclic cascade involving ubiquitination/de-ubiquitination of proteins. To purify isopeptidase, we first constructed a

suitable assay system. This involves a chemical synthesis of a ubiquitin-lysozyme conjugate. The procedure used includes: (1) trypsin-catalyzed transpeptidation reaction using large excess of gly-gly-OEt to selectively modify the C-terminus of ubiquitin to an ethyl ester. (2) The ubiquitin ethyl ester is treated with hydrazine, dialyzed, and converted to ubiquitin azide by treatment with nitrous acid. (3) Addition of lysozyme in triethylamine allows displacement of the azide by the primary amines of lysozyme forming the ubiquitin-lysozyme conjugate. Although the synthetic procedure is relatively straightforward, a complication was found due to, in part, the ubiquitin conjugated lysozyme aggregate easily.

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 an enzyme suicide substrate. Specifically, the work on ubiquitination/deubiquitination should lead to the understanding of this covalent modification cyclic cascade and also shed light on the nature of disease processes in which abnormal metabolism of ubiquitinated protein has been implicated in Alzheimer's disease. Studies on the effect of oscillating transmembrane potential on the function of membrane proteins will lead to our understanding of how oscillating signals work in regulating physiologically important processes such as heartbeat.

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 purify and characterize the enzyme components involved in the ubiquitination/deubiquitination cascade. This involves first the purification and characterization of the isopeptidase(s).

(3) Experimental techniques will be improved for studying the oscillating field effect on cells such as neuroblasts, erythrocytes, and *E. coli*. With the improved techniques, one should be able to monitor electric field-induced conformational transition of membrane bound proteins.

(4) To further explore the roles of enzyme-enzyme complex in the transfer of metabolites.

Publications:

Westerhoff, H. V., Tsong, T. Y., Chock, P. B., Chen, Y., and Astumian, R. D.: How enzymes can capture and transmit free energy contained in an oscillating electric field. *Proc. Natl. Acad. Sci. U.S.A.* 83: 4734-4738, 1986.

Astumian, R. D., Chock, P. B., and Tsong, T. Y.: Enzymes can capture energy from an oscillating electric field for doing essential work of life. J. Electrochem. Soc. 133: 124, 1986.

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, Vol. 17, New York, Academic Press, 1986, pp. 21-42.

Astumian, R. D., Chock, P. B., Tsong, T. Y., Chen, Y., and Westerhoff, H. V.: Can free energy be transduced from electrical noise? Proc. Natl. Acad. Sci. U.S.A. 84: 434-438, 1987.

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Astumian, R. D., Chock, P. B., Chauvin, F., and Tsong, T. Y.: Electroconformational coupling and the effects of static and dynamic electric fields on membrane transport. In Blank, M. and Markov, M. (Eds.): Electromagnetic Fields and Biomembranes, New York, Plenum Press, 1987, in press.

Astumian, R. D., Chock, P. B., Westerhoff, H. V., and Tsong, T. Y.: Energy transduction by electroconformational coupling. In Chock, P. B., Huang, C. Y., Tsou, L., and Wang, J. H. (Eds.): Enzyme Dynamics and Regulation, Amsterdam, Springer-Verlag, 1987, in press.

Shacter, E., Stadtman, E. R., Jurgensen, S. R., and Chock, P. B.: The role of cAMP in cyclic cascade regulation. Meth. Enzymol., 1987, in press.

Shacter, E. and Chock, P. B.: Covalent interconversion and thermostability of proteins. In Henle, K. J. (Ed.): CRC Monograph on Thermotolerance and Thermophily: Observations and Mechanisms, 1987, in press.

Chock, P. B., Jurgensen, S. R., Rhee, S. G., Stadtman, E. R., and Vandenheede, J. R.: The role of cyclic cascades in metabolic regulation. In Chock, P. B., Tsou, L., and Huang, C. Y. (Eds.): Dynamic of Soluble and Immobilized Enzymes. Amsterdam, Springer-Verlag, 1987, in press.

Chock, P. B., Rhee, S. G., and Stadtman, E. R.: Regulation of glutamine synthetase in *E. coli*. In Hervé, G. (Ed.): Allosteric Enzymes, 1987, in press.



2.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00203-14 LB

PERIOD COVERED
October 1, 1986 to September 30, 1987

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 Volunteer LB, NHLBI

COOPERATING UNITS (if any)

Jayasree Nath, Department of Hematology, Walter Reed Army Institute of Research

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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| 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 studies on oxidative processes associated with neutrophil respiratory burst. We have previously partially characterized a novel reaction in which labeled tyrosine is incorporated into high molecular weight material during neutrophil activation. Preliminary studies had suggested that dityrosine was formed but we lacked the authentic compound for comparative studies. In collaboration with Dr. Lin Tsai, dityrosine has been synthesized, purified and characterized. Labeled material was then isolated from neutrophils activated with PMA in the presence of labeled tyrosine and subjected to acid hydrolysis, Dowex 50 chromatography and reversed phase HPLC. Results from these experiments revealed that dityrosine is made by activated neutrophils but no labeled tyrosine is associated with the dityrosine peak. These data indicate that dityrosine which is formed is derived from endogenous unlabeled tyrosine and is independent of labeled tyrosine incorporation into high molecular weight material. Although the chemical form of labeled tyrosine incorporated has not yet been identified, it is alkali stable and acid labile.

Other studies with neutrophils have indicated that cytoplast preparations which are largely depleted of myeloperoxidase-bearing granules generate protein carbonyl groups following stimulation with PMA. These experiments demonstrate that this model can be used to further study enzyme inactivation during neutrophil respiratory burst.

Preliminary studies undertaken with Dr. Donita Garland and Dr. Edward DeMoll have indicated that purified bioactive peptides may be used to compare oxidation products from _in vitro_ and _in vivo_ MFO systems by fast atomic bombardment mass spectroscopy analysis.

Project Description

Objectives:

In earlier studies, we have demonstrated that oxidatively modified proteins accumulate during neutrophil activation, aging and gliosis. This oxidation is likely mediated by mixed-function oxidation (MFO) systems in vivo in reactions similar to those we have described using isolated MFO systems in vitro. In an effort to understand the possible physiological role of protein oxidation, we have continued to study these systems. In addition, we have investigated other oxidative processes associated with neutrophil respiratory burst.

Major Findings:

Tyrosine incorporation. We have previously described a novel reaction in which [$^{14}$C]tyrosine is incorporated into TCA precipitable material of neutrophils stimulated with PMA. Preliminary experiments based on amino acid analysis suggested that the high molecular weight material was protein and that dityrosine was formed. However, at that time authentic dityrosine was not available for comparative studies.

In collaboration with Dr. Lin Tsai, dityrosine was synthesized, purified and characterized by TLC, HPLC and mass spectroscopy. In addition, the UV spectral and fluorescent properties of the dityrosine were determined in a variety of solvents. With the known dityrosine sample, we anticipitated that identification of [$^{14}$C]tyrosine in stimulated neutrophils would be a straightforward process. When the [$^{14}$C]-labeled TCA precipitable material from PMA-stimulated neutrophils was subjected to acid hydrolysis, Dowex chromatography and reversed phase HPLC analysis, the [$^{14}$C]tyrosine was recovered but no label was present in the dityrosine peak. Dityrosine was indeed made by activated neutrophils but it was not labeled. Control, unstimulated cells exhibited no dityrosine. These results indicate that dityrosine which is made is derived from unlabeled endogenous tyrosine and is independent of the incorporation of [$^{14}$C]tyrosine into high molecular weight material. Experiments with oil-LPS or phagocytic stimuli yield similar results. When labeled material was hydrolyzed in 6 N $NH_4OH$ or subjected to exhaustive digestion with neutral proteases, clarified, filtered and analyzed on HPLC, multiple peaks of [$^{14}$C] material were eluted but not one of the peaks could be identified as tyrosine, dopa or dityrosine by retention time or spectral properties. When peaks were individually collected, treated with 6 N HCl at 100°C for 5 minutes, [$^{14}$C] material was obtained which exhibited the same retention time and spectral properties as authentic tyrosine. The elution profile of the labeled material treated with either alkali or neutral proteases was not at all reproducible. SDS electrophoresis and gel filtration reveal [$^{14}$C] label in material over the entire separation range indicating that [$^{14}$C]tyrosine is incorporated into material which is not very homogeneous.

These data prompted a reinvestigation of the [$^{14}$C]tyrosine incorporation reaction. When neutrophils are stimulated with PMA, [$^{14}$C]tyrosine but not labeled alanine, phenylalanine, leucine, or histidine is incorporated into high molecular weight material. Although the tyrosine analog N-acetyltyrosine

competes with tyrosine incorporation, O-methyl tyrosine does not, suggesting that the hydroxyl group is important in this reaction. However, neither serine nor threonine is incorporated under the same conditions suggesting that there is a relatively high specificity for a hydroxyl group of a tyrosine derivative.

The reaction is blocked by azide phenylhydrazine and sodium sulfite but not by aminotriazole. When neutrophils are stimulated with PMA in an atmosphere of $N_2$, no incorporation of tyrosine occurs. The reaction is inhibited by DTT, cysteine, ascorbate, cysteamine, thiourea, but not by mannitol, catalase, or SOD. the [$^{14}$C]tyrosine incorporation occurs readily in PMA-stimulated myeloperoxidase-deficient cells but not in CGD neurtrophils. Taurine, EDTA and EGTA have no effect.

Although previous experiments indicated that [$^{14}$C]-labeled material was not solubilized by mild treatment with 1 N HCl or 1 N NaOH, prolonged acid hydrolysis (6 N HCL, 100°C, 18-24 hours; 6 N HCl 155°C, 45' min) also solubilized the labeled tyrosine. Following acid hydrolysis, the labeled material bound to Dowex could be eluted with 2 N $NH_4OH$ and both the retention time and spectral properties were identical to authentic tyrosine.

Taken together, these data suggest that an intact NADPH-oxidase pathway or some component of activation is required for the incorporation of [$^{14}$C]tyrosine into high molecular weight material. Currently, we have no evidence for the formation of dityrosine from labeled tyrosine nor any evidence that [$^{14}$C]tyrosine is linked directly to protein.

Cytoplast preparations. In order to study neutrophil-mediated oxidation of proteins in the absence of myeloperoxidase chlorination reactions, cytoplasts were prepared and stimulated with PMA. Results from these exerpiments indicate that stimulated cytoplasts generate protein carbonyl derivatives and this reaction is likely due to the activated NADPH oxidase.

Peptides. Several peptides of known sequence were purified by reversed phase HPLC and these peptides were treated with a nonenzymic MFO system. Following treatment, the peptides were reisolated by reversed phase HPLC and analyzed for alterations in spectral properties and incorporation of [$^3$H]$NaBH_4$. Aliquots of the unlabeled modified and control peptides were analyzed by FAB mass spectroscopy. Results from these experiments indicate that oxidative modifications can be identified by this method.

Proposed Course:

Tyrosine incorporation into activated neutrophils. Further studies are designed to determine the chemical form of tyrosine into high molecular weight material during neutrophil activation in order to ascertain whether this reaction is nonspecific or whether it has some physiological role in neutrophil activation. We plan to examine the possibility that the tyrosine hydroxyl group is involved in an acid labile ester or glycosidic bond.

Although dityrosine formation in activated neutrophils in independent of [$^{14}$C]tyrosine incorporation, the possibility exists that dityrosine cross-links

may be generated in immune complexes and matrix proteins. In this case, dityrosine may render proteins resistant to proteolysis and thus contribute to the accumulation of protein fragments observed in synovial fluid from patients with rheumatoid arthritis. We plan to examine immune complexes, matrix proteins, and synovial fluid for the presence of dityrosine.

Studies with cytoplast preparations. As noted above, preliminary studies indicate that carbonyl generation occurs in stimulated neutrophil cytoplast preparations suggesting that the activated NADPH oxidase (which is membrane associated and present in cytoplasts) plays a physiological role in this process. These studies suggest that cytoplasts represent a good model for the enzyme inactivation which is mediated by activated neutrophils.

Peptide studies. We plan to characterize the MFO-mediated oxidative modifications of several bioactive peptides of known sequence by FAB mass spectroscopy and use these peptides for comparative studies with various enzymic, nonenzymic and cell-mediated MFO systems.

Publications:

Oliver, C. N.: Inactivation of enzymes and oxidative modification of proteins by stimulated neutrophils. Arch. Biochem. Biophys. 253: 62-72, 1987.

Oliver, C. N., Ahn, B.-W., Moerman, E. J., Goldstein, S., and Stadtman, E. R.: Age-related changes in oxidized proteins. J. Biol. Chem. 262: 5488-5491, 1987.

Starke, P. E., Oliver, C. N., and Stadtman, E. R.: Modification of hepatic proteins in rats exposed to high oxygen concentrations. FASEB J. 1, 1987, in press.

Oliver, C. N., Levine, R. L., and Stadtman, E. R.: A role of mixed-function oxidation reactions in the accumulation of altered enzyme forms during aging. J. Am. Geriatric Soc., 1987, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00204-20 LB

PERIOD COVERED
October 1, 1986 to September 30, 1987

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: | John R. Jefferson | Staff Fellow (6/22/86-) | LB, NHLBI |
| | Gerard J. O'Donnell | Chemist, M.D. (9/14/86- ; GS-9) | LB, NHLBI |

COOPERATING UNITS (if any) M.R. Maurizi, Lab. Molecular Biology, NCI; J.B. Hunt, NSF (Chem. Div.); Susan Green and P. Hensley, Georgetown Univ.; A. Shrake, Bur. Biologics; H.K. Schachman, Univ. of California, Berkeley; D. Eisenberg, Univ. 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 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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☐ (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 (GS) from *E. coli*. Various analogs of ATP substituted at the 6- or 8-position of the purine ring were further modified with spectrophotometric and fluorometric probes or an electron dense Pt(II) marker. Fluorescence energy transfer measurements showed that active site nucleotide probes in the dodecamer are separated by ~ 56 Å. The enzyme from *S. typhimurium* also has been labeled at active sites or adenylylation sites with mercaptonucleotide•platinum(II) for crystallization and X-ray structural analysis in D. Eisenberg's laboratory at UCLA. Nucleotide probes also are being used for determining the relative intra-subunit distances between the active sites and Trp 57 and Trp 158.

(2) Calorimetric studies of $Zn^{2+}$-induced stacking of GS dodecamers have shown that $\Delta Cp \simeq -850$ cal/K•mol, suggesting a dominant role of water in the polymerization process. Thermodynamic parameters for binding various active-site ligands to *E. coli* GS also have been obtained by calorimetry and fluorometry.

(3) The octameric GS from bovine brain was found to contain two essential divalent cation sites/subunit--a structural site and a higher affinity nucleotide-metal ion site which is filled after the first site is occupied by Mn(II) or Mg(II). Although the enzyme is active with either Mg(II) or Mn(II) *in vitro*, Mg(II) is bound to the brain enzyme *in vivo*. Allosteric sites for chloride and L-glutamate could regulate activity.

(4) Regulatory subunits of *E. coli* aspartate transcarbamoylase bind Zn(II) with high affinity and kinetic constants for this interaction are being determined.

(5) Possible roles of Zn(II) and Mn(II) ions in maintaining quaternary structures of arginases from yeast and bovine liver are being investigated.




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

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.

(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 were 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.

(5) To study metal ion binding to arginase from yeast and bovine liver in order to understand the roles of divalent cations in stabilizing the quaternary structures of these enzymes.

Major Findings:

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

*E. coli* GS is composed of 12 identical subunits ($M_r$ 51,800) 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 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

58.

substitute for ATP in the auto-inactivation reaction of Mn•GS with L-met-S-sulfoximine at pH ~ 7 (J. Biol. Chem. 257, 4271-4278, 1982). With L-met-S-sulfoximine phosphate, 2 $Mn^{2+}$, and ADP ($K'_A > 10^{12}$ $M^{-1}$) tightly bound at a subunit active site both intra- and inter-subunit contacts are strengthened (J. Biol. Chem. 257, 4271, 7246, 1982; Curr. Top. Cell. Regul.: Mol. Interactions 26, 191-206, 1985). In order to determine how active-site ligand binding affects the structural stability of GS and how interactions between active sites or between active sites and other protein groups occur, we have introduced various structural probes into active sites of the enzyme (Maurizi and Ginsburg, Biochemistry 25, 131-140, 1986).

The results obtained in this laboratory are entirely consistent with the atomic model at 3.5 Å resolution for glutamine synthetase from S. typhimurium recently proposed by R.J. Almassy, C.A. Jason, R. Hamlin, N.-H. Xuong, and D. Eisenberg (Nature 323, 304-309, 1986). An unusual feature of the enzyme structure is that the 12 active sites are formed at heterologous interfaces between subunits arranged within a hexagonal ring of two face-to-face eclipsed rings. The 2 $Mn^{2+}$ at each subunit are in the subunit C-terminal domain, whereas the nucleotide binding site is in the N-terminal region of an adjacent subunit. (H.B. Pinkofsky, A. Ginsburg, I. Reardon, and R.L. Heinrikson, J. Biol. Chem. 259, 9616-9622, 1984). This explains how the binding of substrates enormously stabilizes quaternary structures of glutamine synthetase. However, the distance between $Mn^{2+}$ at neighboring active sites within the same ring of 6 subunits is ~ 45 Å (Almassy et al.) which is less than the 56 Å between nucleotide probes estimated in this laboratory using fluorescence energy transfer measurements (Maurizi et al., Biochemistry 25, 141-151, 1986). Apparently, the adenosine moiety of an ATP molecule binds more towards the exterior surface of the enzyme than do the $Mn^{2)}$ ions. Also, the atomic model places the subunit adenylylation site ~ 22 Å from the $Mn^{2+}$, which is farther than that indicated by previous measurements. In attempts to solve these apparent discrepancies, we have introduced an electron dense probe into each active site of unadenylylated glutamine synthetase from S. typhimurium and have sent these derivatives to the laboratory of D. Eisenberg for X-ray structural analyses. The electron dense probes consisted of coordination complexes between 8-mercapto-ADP and 6-mercaptopurine ribonucleoside diphosphate and aquo glycyl-L-methioninato platinum(II). Our first enzyme derivatives did not crystallize properly. However, we plan to remake these derivatives with HPLC-purified enzyme since an impurity in the protein was found later. Also, we have introduced aquo glycyl-L-methioninato platinum(II) into glutamine synthetase from S. typhimurium after adenylylation with 6-mercaptopurine ribonucleoside triphosphate. Crystals of this HPLC-purified derivative are slowly growing but have not yet attained the desired size.

At least 1 of 2 tryptophanyl residues in each subunit was found to be very near the nucleotide binding site as evidenced by changes in tryptophanyl residue fluorescence on binding ATP, mercaptonucleotides, or other ATP analogues. Collaborative studies with Dr. Jay Knutson using time-resolved fluorometry are expected to give information on intra-subunit interactions. We hope to be able to resolve Trp 57 from Trp 158 by fluorescence quench techniques together with time-resolved fluorometry using a global (multiple wavelength) approach developed by Dr. Jay Knutson. Initial analysis of the intrinsic tryptophanyl residue

fluorescence in E. coli glutamine synthetase gave 3 life times of ~ 4.8, 2.3, and 0.5 ns.

(2) Thermodynamics of $Zn^{2+}$-induced stacking of glutamine synthetase (GS) dodecamers.

The stacking of GS dodecamers of $M_r$ 622,000 from E. coli has been studied by calorimetry in order to investigate the forces governing macromolecular self-assembly reactions (unpublished results of A. Ginsburg and M. Blackburn). $Zn^{2+}$ binds to a site distinct from the active site of each subunit with $K_A = 5 \times 10^6$ $M^{-1}$ at pH 7.0 and deforms the enzyme in such a way that when 50 mM $MgCl_2$ also is present, spontaneous face-to-face aggregation of GS dodecamers and later side-to-side interactions between twisted single strands occur. GS with active sites blocked by inactivation with $Mn^{2+}$, L-Met-S-sulfoximine and ATP was used for these studies in order to assure that $Zn^{2+}$ was not binding to active sites of the enzyme. Kinetic studies of $Zn^{2+}$-induced polymerization of GS along the 6-fold axes of symmetry indicate that the reaction rate increases with increasing temperature with an Arrhenius activation energy of 17.7 kcal/mol of dodecamer. Enthalpy changes ($\Delta H$) for (1) the binding of $Zn^{2+}$ to inactivated GS with accompanying proton release (1 eq $H^+/Zn^{2+}$ bound) and protein conformational changes, and (2) the $Zn^{2+}$-induced aggregation of GS dodecamers and, as confirmation, (3) the reversal of reactions in (1) and (2) by addition of the $Zn^{2+}$ chelator pyridine-2,6-dicarboxylate were measured by calorimetry at pH 7.0. A batch calorimeter (LKB 10700) equipped with gold cells and a microtitrator (LKB 2107-350) was used at 22.5, 30 and 38°C with the detector on a 10 μV scale. In the absence of $MgCl_2$, the addition of 0.66 eq of $Zn^{2+}$/subunit produces no aggregation of GS (as monitored by light scattering) and $\Delta H$ = 115 kcal/mol of GS dodecamer for (1) at 38°C with $\delta \Delta H/\delta T \approx 1500$ cal/K·mol for $Zn^{2+}$ binding. Subtracting the latter value from that obtained for (1) + (2) and averaging the corresponding difference in values from the reversal of reactions (3) gave $\Delta Cp \approx -850$ cal/K·mol for protein-protein interactions in (2). The large $\Delta Cp$ value for (2) implicates a dominant role of water in the stacking process. Accordingly, pressure effects on the stacking of GS dodecamers are currently being investigated.

(3) Thermodynamics of active-site ligand binding to E. coli glutamine synthetase. (A. Ginsburg, E.G. Gorman, S.N. Neece, and M.B. Blackburn, Biochemistry 26, 1987, in press.)

Active-site ligand interactions with dodecmeric glutamine synthetase were studied by calorimetry and fluorometry using the nonhydrolyzable ATP analogue adenylyl-imidodiphosphate (AMP-PNP), L-glutamate, L-Met-(S)-sulfoximine, and the transition state analogue L-Met-(S)-sulfoximine-phosphate. Measurements were made with the unadenylylated enzyme at pH 7.1 in the presence of 100 mM KCl and 1.0 mM $MnCl_2$, under which conditions the two catalytically essential metal ion sites per subunit are occupied and the stoichiometry of active-site ligand binding is equal to 1.0 equiv/subunit. Thermodynamic linkage functions indicate that there is strong synergism between the binding of AMP-PNP and the L-Met-(S)-sulfoximine ($\delta \Delta G'$ = -1.5 kcal/mol). In contrast, there is a small antagonistic effect between the binding of AMP-PNP and L-glutamate ($\delta \Delta G'$ = +0.3 kcal/mol).

Thermodynamic parameters for binding various substrates and substrate analogues to glutamine synthetase are useful for mapping interactions at active sites. L-Glutamine and L-Met-(S)-sulfoximine interact with the L-glutamate and ammonia subunit sites and both produce an ordering effect ($\Delta S \approx -21$ cal/K·mol) with large negative $\Delta H$ values (without significant net proton uptake). In contrast, the binding of L-glutamate is less exothermic and the apparent ordering effect (as judged by the negative $\Delta S$ value) is less, possibly due to charge neutralization and changes in hydration waters. The binding of ADP is exothermic as is the binding of $P_i$ to the ADP·Mg·enzyme (Shrake et al. in this laboratory, 1977; 1978). In contrast, the binding of ATP or AMP-PNP is endothermic. Also, the binding of $P_i$ to the ADP·Mg·enzyme·Glu complex is entropically dominated at 303K. Similarly, the binding of AMP-PNP and L-glutamate to the Mn·enzyme at 303K is entropically controlled. The transition state analogue L-Met-(S)-sulfoximine-phosphate spans the active site protein groups responsible for binding L-glutamate, ammonia, and the $\gamma$-phosphate of ATP. The free energy change for binding L-Met-(S)-sulfoximine-phosphate is very exergonic and both enthalpy and entropy changes are favorable and are similar to those observed for L-Met-(S)-sulfoximine and ATP binding, respectively. Thus, the thermodynamic parameters obtained for binding the transition state analogue appear to reflect those obtained for ligand binding to the subunit sites responsible for binding L-glutamate, ammonia, and the $\gamma$-phosphate of ATP.

The thermodynamic parameters for active-site ligand binding to *E. coli* glutamine synthetase contain contributions from both protein-ligand and protein-protein interactions. The positive entropy change for interaction of the enzyme with a full complement of substrates could result from forcing the nucleotide into a more hydrophobic environment for interaction with the $\gamma$-carboxylate group of L-glutamate and concomitant changes in hydration. However, the ionic interactions of the $\gamma$-phosphate of ATP play a major role in contributing to the entropy changes observed. Also, interactions at the L-glutamate and ammonia sites produce ordering effects that are opposed by large exothermic heats of binding. Active-site ligand stabilization of intra- and inter-subunit bonding domains to produce a more compact dodecameric structure would be expected to lead to negative values of $\Delta H$ and $\Delta S$ through the strengthening of hydrogen bonds in the protein interior and van der Waals' interactions brought about by the partial withdrawal of nonpolar groups from water. These more remote stabilizing effects of active-site ligand binding appear to be offset by local charge neutralization effects at active sites.

(4) ADP, chloride ion, and metal ion binding to bovine brain glutamine synthetase (GS). (Investigators: M.R. Maurizi, H.B. Pinkofsky, P.J. McFarland, and A. Ginsburg.)

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.

Starting from fresh or frozen bovine brain, we were able to purify glutamine synthetase with specific activities in the biosynthetic and the $\gamma$-glutamyl transfer reactions higher than those reported previously. The absorption coefficient ($A^{0.1\%}_{280nm,1cm} = 1.50 \pm 0.05$) was determined by refractometry and by

quantitative amino acid analysis to allow measurement of the absolute enzyme subunit concentration. We then began a systematic examination of the binding affinities and stoichiometries of binding for nucleotides, metal ions, and anions interacting with the enzyme. Kinetic titrations were conducted to relate the binding properties of the different ligands to their effects on the enzymatic activity of the enzyme. We found no evidence for negative cooperativity or for half-of-the-sites reactivity with any of the ligands examined.

The purified enzyme is free of metal ions and can bind reversibly up to 2 $Mn^{2+}$ or $Mg^{2+}$ per subunit which is consistent with our previous finding of 2 $Mn^{2+}$ bound per subunit in the presence of ADP and the transition state analogue L-met-S-sulfoximine phosphate (M.R. Maurizi, H.B. Pinkofsky, P.J. McFarland, and A. Ginsburg, Arch. Biochem. Biophys. 246, 494-500, 1986). In those studies, we also obtained evidence that $Mg^{2+}$ is bound to brain GS *in vivo*.

In ADP-supported γ-glutamyl transfer at pH 7.2, kinetic analyses of saturation functions gave $[S]_{0.5}$ values of ~ 1 μM for $Mn^{2+}$, ~ 2 mM for $Mg^{2+}$, 19 nM for ADP•Mn and 7.2 μM for ADP•Mg. The method of continuous variation applied to the $Mn^{2+}$-supported reaction indicated that all subunits of the purified enzyme express activity when 1.0 eq of ADP is bound per subunit. Measurements of equilibrium binding of $Mn^{2+}$ to the enzyme in the absence and presence of ADP were consistent with each subunit binding free $Mn^{2+}$ ($K_A' \simeq 1.5 \times 10^5\ M^{-1}$) before binding the Mn•ADP complex ($K_A' \simeq$ 1.[illegible] $\times 10^6\ M^{-1}$). The binding of the first $Mn^{2+}$ or $Mg^{2+}$ to each subunit produces structural perturbations in the octameric enzyme, as evidenced by UV spectral and tryptophanyl residue fluorescence changes. The enzyme therefore has one structural site per subunit for $Mn^{2+}$ or $Mg^{2+}$ and a second site per subunit for the metal ion-nucleotide complex, both of which must be filled for activity expression. Chloride binding ($K_A' \sim 10^4\ M^{-1}$) to the enzyme was found to have a specific effect on the protein conformation -- producing a substantial (30%) quench of tryptophanyl fluorescence and increasing the affinity of the enzyme 2- to 4-fold for $Mg^{2+}$ or $Mn^{2+}$. Arsenate, which activates the γ-glutamyl transfer activity by binding to an allosteric site, and L-glutamate also cause conformational changes similar to those produced by Cl binding. Anion binding to allosteric sites and divalent metal ion binding at active sites both produce tryptophanyl residue exposure and tyrosyl residue burial without changing the quaternary enzyme structure.

(5) $Zn^{2+}$ release and uptake by regulatory subunits of *E. coli* aspartate transcarbamoylase. (Investigators: J.R. Jefferson, J.B. Hunt, and A. Ginsburg.)

Rates of $Zn^{2+}$ release and uptake by regulatory subunits ($r_2$) of aspartate transcarbamoylase (ATCase; $c_6r_6$) have been studied in $K\text{-}PO_4$, pH 7.0 buffer, using the metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR). $Zn^{2+}$ is tetrahedrally bound by the 4 thiols of each r chain and is released upon the addition of 1 eq/-SH of p-hydroxymercuriphenyl sulfonate (PMPS). $Zn^{2+}$ is rebound to r dimers when excess 2-mercaptoethanol (2-ME) is added to displace PMPS from the protein and $r_2$ then can combine with catalytic subunits ($c_3$) for ATCase assembly (J.B. Hunt, S.H. Neece, and A. Ginsburg, Anal. Biochem. 146, 150-157, 1985). Stopped-flow measurements at 500 nm of PMPS-promoted $Zn^{2+}$ release from regulatory subunits was first order with a half-time of 50 ± 3 ms at 15°C and was independent of [PMPS]. This rate is > 2800-fold faster than

the corresponding rate of $Zn^{2+}$ release from intact ATCase, which is dependent on the concentration of PMPS (Hunt et al., J. Biol. Chem. 259, 14793-14803, 1984).

In using PAR as an indicator for $Zn^{2+}$ uptake, complex equlibria are involved, which must be coupled to the binding of $Zn^{2+}$ to the acceptor:

$$(PAR)_2Zn \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} PAR\text{-}Zn \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} PAR + Zn^{2+}$$

The accelerating effect of added 2-ME was eliminated by extrapolation to zero [PAR], suggesting that 2-ME simply shifts the above equilibria to the right. More experiments will be necessary to establish whether or not the PAR-$Zn^{2+}$ signal can be used to determine the intrinsic k for $Zn^{2+}$ binding to $r_2$ subunit. After quickly separating the protein-PMPS adduct from free $Zn^{2+}$ and treating the protein-mercaptide with excess 2-ME, PAR-$Zn^{2+}$ was used to donate $Zn^{2+}$ to regulatory subunits. Rates of $Zn^{2+}$ uptake by regulatory subunits were studied as a function of [PAR] and [ME-2]. Rates of $Zn^{2+}$ binding to $r_2$ were inversely dependent on [PAR] and extrapolation of 2nd order rate constants gave $\sim 10^6$ $M^{-1}s^{-1}$ as a lower limit for the reaction of regulatory subunits with $Zn^{2+}$ at 15°C in the absence of PAR. It is unlikely, therefore, that the binding of $Zn^{2+}$ to $r_2$ subunit is rate-limiting during ATCase assembly since the rate of formation of r:c bonding domains during the ATCase assembly is $\sim 6 \times 10^5$ $M^{-1}s^{-1}$ (M.A. Bothwell and H.K. Schachman, J. Biol. Chem. 255, 1971, 1980). ATP (0.1 mM) and CTP (0.2 mM), allosteric effectors of ATCase, produced only ~ a 1.4-fold increase in the rate of $Zn^{2+}$ binding to regulatory subunits.

(6) Arginase from bovine liver. (Investigators: J.R. Jefferson, G.J. O'Donnell, and A. Ginsburg.)

Last year, we reported the discovery of 1 $Zn^{2+}$/subunit in the trimeric arginase purified from yeast by Susan Green (a graduate student of P. Hensley at Georgetown University). In addition, arginases from yeast and mammalian tissues are activated by $Mn^{2+}$ and we identified 1 site/subunit in yeast arginase for binding $Mn^{2+}$ ($K_A' \simeq 6 \times 10^4/M^{-1}$).

In order to determine if $Zn^{2+}$ has a role in maintaining the quaternary structure of liver arginase (which has been reported to be a tetramer), we have purified this enzyme from bovine liver. Also, we are investigating the activation of this enzyme by $Mn^{2+}$. Incubation of the enzyme with EDTA inactivates liver arginase and activity can be restored by addition of excess $Mn^{2+}$. Including 1 mM $MnCl_2$ in the purification steps also was found to be necessary for stabilization of arginase activity. In the next to the last gel filtration step of the purification procedure, the enzyme activity was relatively stable and we found 2.5 equiv of $Zn^{2+}$ per tetramer of $M_r$ = 120,000. In the last gel filtration step, the enzyme activity was unstable and the $Zn^{2+}$ content decreased to ~ 0.4 equiv/tetramer. We have used this latter fraction to immunize a rabbit and have just obtained antisera which has high levels of precipitating anti-arginase immunoglobulins (Ig; anti-argase Ab) not present in the prebleed serum. Arginase activity is not inhibited by the interaction with anti-Argase antibodies.

Our preparation of purified arginase had two active forms, as detected on nondenaturing polyacrylamide gel electrophoresis. However, gel electrophoresis of the arginase-Ig complex in the presence of sodium dodecyl sulfate showed only one arginase protein band at ~ 36,000 $M_r$. All $Zn^{2+}$ and arginase activity had been precipitated by the rabbit anti-Argase Ab. The anti-Argase Ab will be used to characterize arginase from freshly broken liver cells in order to determine (1) if multiple active forms arise from proteolysis (despite the addition of protease inhibitors during purification steps) and (2) if $Zn^{2+}$ is present before the enzyme becomes damaged by sulfhydryl group oxidation.

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 analogues), and inhibitors with GS from *E. coli* and from bovine brain. To locate specific sites in the subunit and the oligomeric structures in order to better understand tertiary and quaternary structural changes that occur on ligand interactions with these enzymes.

(3) The thermodynamics of $Zn^{2+}$-induced face-to-face aggregation of GS dodecamers will be studied in order to investigate the forces governing macromolecular self-assembly reactions.

(4) To further characterize the reversible thermal transition of dodecameric GS from *E. coli* (see last year's Annual Report).

(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 regulatory subunits will continue in order to better understand the mechanism of assembly of ATCase from catalytic and regulatory chains.

(6) A possible role of $Zn^{2+}$ in maintaining the quaternary structures of bovine liver and yeast arginases is being investigated in addition to studies on the activation of these enzymes by $Mn^{2+}$. We also have planned a calorimetric study of ligand interactions and protein-protein interactions responsible for the multienzyme complex formed between arginase and ornithine transcarbamoylase from yeast.

Publications:

Maurizi, M.R., Pinkofsky, H.B., McFarland, P.J., and Ginsburg, A.: $Mg^{2+}$ is bound to glutamine synthetase extracted from bovine or ovine brain in the presence of L-methionine-S-sulfoximine phosphate. Arch. Biochem. Biophys. 246: 494-500, 1986.

Maurizi, M.R., Pinkofsky, H.B., and Ginsburg, A.: ADP, chloride ion, and metal ion binding to bovine brain glutamine synthetase, Biochemistry 26, in press, 1987.

Ginsburg, A., Gorman, E.G., Neece, S.H., and Blackburn, M.B.: Thermodynamics of active-site ligand binding to Escherichia coli glutamine synthetase, Biochemistry 26, in press, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00205-32 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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)

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

Others: Rong-Xin Zhu — Fogarty Visiting Fellow (Terminated July 2, 1987) — LB, NHLBI

Joe Nathan Davis — Laboratory Research Assistant — LB, NHLBI

COOPERATING UNITS (if any)

Gas Research Institute, Chicago, Illinois.

Dr. August Böck, University of München, München, West Germany.

Dolph Hatfield, National Cancer Institute, NIH.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.1 | 2.00 | 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 selenium-dependent clostridial glycine reductase that synthesizes ATP concomitant with the reductive deamination of glycine was investigated with emphasis on (1) large-scale isolation of the essential enzyme components to be used for reaction mechanism studies and (2) mechanism of biosynthesis of the selenoprotein component (protein A) of the reductase complex. A reproducible protocol for the isolation of protein C in apparently homogenous form was developed using hydrophobic chromatographic procedures. The resulting material, enriched about 1000-fold, is exceedingly stable and can be stored many months at -20°C with no detectable loss of activity. The mechanism of insertion of a selenocysteine residue into selenium-dependent enzymes also was studied in a collaborative project with August Böck's group in München using a formate dehydrogenase in Escherichia coli as experimental material. This very oxygen-labile enzyme, which had not previously been isolated, was purified extensively using the NIH anaerobic laboratory. The membrane-bound enzyme was solubilized and enriched using hydrophobic chromatographic procedures. After many difficulties we finally have devised a procedure for isolation of a selenocysteine peptide from the purified selenium-containing formate dehydrogenase for sequence analysis. The gene encoding this protein contains a TGA stop codon which is the presumed codon specifying insertion of the selenocysteine residue but this has to be proven by amino acid sequence of the gene product. Site specific mutagenesis of the TGA codon to TGC or TGU (cysteine codons) by Böck's group eliminated the selenium requirement for formate dehydrogenase expression. The resulting enzymes in which cysteine replaced selenocysteine exhibited some catalytic activity. The cysteine-containing enzyme was partially purified using the same procedures developed for isolation of the selenocysteine-containing species. Precise comparison of catalytic activities of Se vs. S enzyme species will be made when highly purified samples are obtained. Replacement of selenocysteine with serine resulted in a completely inactive enzyme.



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

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. Development of a revised procedure for large-scale isolation of protein B. 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 Methanosarcina barkeri and Methanococcus vannielii enzymes involved in methane biosynthesis from acetate and from $C_1$ compounds. (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 seleno-tRNAs and selenoenzymes.

   (a) Identification of selenium donor and mechanism of biosynthesis of 2-selenouridine residues in bacterial tRNAs. Characterization of the enzymes involved in the process.

   (b) Identification of the protein factors and the tRNA species involved in insertion of selenocysteine into proteins in response to the UGA termination codon.

3. Isolation, characterization, and 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) Synthesis of ATP by the selenium-dependent clostridial glycine reductase complex requires the participation of three soluble proteins, selenoprotein A and proteins B and C. Employing a simplified isolation procedure, highly stable preparations of protein C that are enriched about 1000-fold have been obtained. The native protein consists of two subunits that partially separate during electrophoresis on native gels and migrate as smearing bands of about 90 K and 110 K. On molecular sieve columns the protein C activity migrates either as a 200 kDa species or one of about twice this size

depending on buffer conditions. On SDS gels two approximately equally stained bands that migrate as 49 K and 58 K subunits are detected. Attempts to separate and recover catalytically active subunits under non-denaturing conditions in order to prove that both are essential for C activity have not been successful. Accordingly, protein isolated from polyacrylamide electrophoretic gels will be used as antigens and the elicited antibodies will be tested as possible inhibitors of C activity to prove this point. The electronic absorption spectrum of purified "C protein" indicates the presence of tyrosine and phenylalanine and lack of tryptophan. There is no convincing evidence of a bound chromophore on the protein.

The project of developing a revised purification procedure for protein B that was assigned to R.-X. Zhu was unproductive. An experiment carried out by the P.I. indicated that a second hydrophobic chromatographic step using octyl-Sepharose would be useful for further purification. Successful exploitation of this procedure appears to require a more experienced investigator and will be attempted later. Although homogeneous preparations of protein B were obtained earlier in small amounts, a procedure for larger scale recovery is needed.

A recently described, improved method for identification of carbonyl groups (such as pyruvate residues) in proteins will be tried with protein B to see if the suspected pyruvate is really present. The necessary preliminary treatment with cyanoborohydride gave the expected results and the $^{3}H$-labeled reagent will be used later.

(b) Studies on enzymes involved in methane biosynthesis from acetate and characterization of carbon monoxide dehydrogenase from methane bacteria were carried out by Dr. David Grahame and Dr. Edward DeMoll. Their studies are described in their separate reports.

2. (a) Only incidental studies on the biochemistry of seleno-tRNAs have been carried out during the past year after the departures of Arthur Wittwer and Wei-Mei Ching. Continued efforts of Lin Tsai to synthesize the selenium analogue of a thio-nucleoside (5-methoxycarbonylmethyl-2-thiouridine) recently isolated from bacterial tRNAs appear to be successful. The selenonucleoside is suspected to be one of the selenonucleosides present in the tRNAs of Methanococcus vannielii and the authentic synthetic compound is needed as a reference substance.

(b) Part of the research efforts in this laboratory have as their aim the elucidation of the mechanism of selenocysteine incorporation into selenium-dependent enzymes. One of our approaches, carried out by Dr. Gregory Garcia (see his independent report) is to isolate the gene from a C. sticklandii cDNA library that encodes the glycine reductase selenoprotein A and, from the DNA sequence, identify the codon that corresponds to the known position of the selenocysteine residue in the gene product.

A similar approach, as a collaborative effort with Dr. August Böck and his group at the University of München, is underway using a formate dehydrogenase from Escherichia coli. In this case the gene has already been isolated, cloned, and sequenced by Böck's group, but the protein chemistry is missing. Of great interest is the finding that a TGA stop codon occurs in the open reading frame of the formate dehydrogenase gene and a number of indirect lines of evidence strongly indicates that TGA (UGA in the mRNA) specifies incorporation of selenocysteine and this occurs as a co-translational event. The recent finding of the same stop codon in the glutathione peroxidase gene, in a position that corresponds to the known location of selenocysteine in the protein, indicates that TGA indeed specifies Se-cys in proteins. As a means of determining how this unusual usage of a stop codon works, an in vitro protein synthesis system is being developed in order that the system can be fractionated and the required factors detected and identified. Details of this approach, using formate dehydrogenase:β-galactosidase gene fusions, are described in the report of Dr. Milton Axley.

Some progress on the protein and peptide chemistry aspects of the E. coli formate dehydrogenase problem has been made. This protein which couples to hydrogenase and is membrane-bound has never been isolated in pure form. Because of its $O_2$ sensitivity I have carried out purification steps in the anaerobic laboratory using hydrophobic chromatographic matrices and active, highly purified preparations have been made. A protein in which the selenocysteine residue has been replaced by cysteine (by conversion of the TGA codon to TGC which specifies cysteine) is also enzymically active and this has been purified by the same procedure.

Slow progress has been made on isolation of a $^{75}$Se-labeled peptide prepared from the $^{75}$Se-labeled formate dehydrogenase in order that the protein and DNA sequences can be lined up to make sure TGA and Se-cysteine positions coincide. Lability of the protein and the generated peptides together with the low amount of protein present has made this a very difficult problem.

In collaboration with Dolph Hatfield, National Cancer Institute, NIH, studies are in progress to determine whether a minor serine tRNA that can serve as an in vitro UGA suppressor may be the tRNA utilized as the selenocysteine carrier. With this particular tRNA, the esterified serine is known to be subsequently phosphorylated and o-phosphoserine aminoacylated to the tRNA might then be modified by a specific enzyme so that the o-phosphate is eliminated and replaced with selenium. Although this particular serine tRNA occurs in eukaryotes, it has not been detected in bacteria but nevertheless is an attractive possible carrier of a Se-cys residue from the chemical point of view.

Proposed Course:

Scale up of purification procedures for proteins B and C of clostridial glycine reductase complex. Characterization of protein C component with respect to composition and catalytic function. Production of antibodies to the two suspected subunits to see if both are essential components of the protein C and required for catalytic activity. Studies of the reconstituted glycine reductase system (proteins B and C plus selenoprotein A) using thiophosphate in place of orthophosphate to gain information concerning the mechanism of ATP synthesis. In this reaction one ATP is synthesized for each mole of glycine reduced to acetate and ammonia. The identity of the protein bound, phosphorylated intermediate that is generated in the redox reaction is of particular interest. Pure proteins B and C will be used as antigens to produce antibodies needed for studies of regulation of biosynthesis of these proteins. In earlier studies it was noted that expression of one or both of these proteins was greatly decreased when millimolar levels of alternative substrates such as proline or pyruvate were added to the culture media. If the cDNA that encodes for the selenoprotein A can be located and cloned, factors that regulate the synthesis of this protein (or its precursor) can be studied in greater detail. Highly active polyclonal antibodies specific for this protein are available from previous work and these are especially useful in such studies. A Schiff base derivative of protein B with p-aminobenzoic acid will be reduced with cyanoborohydride and examined for presence of a derivative with pyruvate.

Continuation of a collaborative research project with Dr. August Böck of München to determine the mechanism of biosynthesis of a selenoprotein. The specific selenoenzyme studied is an E. coli formate dehydrogenase in which there is an 80 K dalton selenocysteine-containing polypeptide subunit. The cDNA coding for this selenopolypeptide has been cloned by the München group and the DNA sequence determined. Procedures for isolation of selenocysteine-containing peptide fragments from $^{75}$Se-labeled protein have been worked out and are in the process of scale up in order to obtain sufficient pure peptide for amino acid sequence analysis. Comparison of cDNA and peptide sequences are needed to substantiate the indirect evidence that TGA specifies Se-cysteine.

Publications:

Hartmanis, M.G.N. and Stadtman, T.C.: Diol Metabolism and Diol Dehydratase in Clostridium glycolicum. Arch. Biochem. Biophys. 245: 144-152, 1986.

Wittwer, A.J. and Stadtman, T.C.: Biosynthesis of 5-Methylaminomethyl-2-Selenouridine, A Naturally Occurring Nucleoside in Escherichia coli tRNA. Arch. Biochem. Biophys. 248: 540-550, 1986.

Zinoni, F., Birkmann, A. Stadtman, T.C., and Böck, A.: Nucleotide Sequence and Expression of the Selenocysteine-Containing Polypeptide of Formate Dehydrogenase (Formate-Hydrogen-Lyase-Linked) of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 83: 4650-4654, 1986.

Grahame, D.A. and Stadtman, T.C.: Carbon Monoxide Dehydrogenase from *Methanosarcina barkeri*: Disaggregation, Purification, and Physicochemical Properties of the Enzyme. *J. Biol. Chem.* 262: 3706-3712, 1987.

Hartmanis, M.G.N. and Stadtman, T.C.: Solubilization of a Membrane-Bound Diol Dehydratase With Retention of EPR *g* = 2.02 Signal by Using 2-(N-Cyclohexylamino)ethane Sulfonic Acid Buffer. *Proc. Natl. Acad. Sci. U.S.A.* 84: 76-79, 1987.

Sliwkowski, M.X. and Stadtman, T.C.: Purification and Immunological Studies of Selenoprotein A of the Clostridial Glycine Reductase Complex. *J. Biol. Chem.* 262: 4899-4904, 1987.

Stadtman, T.C.: Bacterial Selenoenzymes and Seleno-tRNAs. In Combs, G.F., Jr., Spallholz, J.E., Levander, O.A., and Oldfield, J.E. (eds.): Selenium in Biology and Medicine, Part A, 1987, pp. 81-89.

Zhu, R.-X., Ching, W.-M., Chung, H.K., Rhee, S.G., and Stadtman, T.C.: Purification of Individual tRNAs Using a Monoclonal Anti-AMP Antibody Affinity Column. *Anal. Biochem.* 161: 460-466, 1987.

DeMoll, E., Grahame, D.A., Harnly, J.M., Tsai, L., and Stadtman, T.C.: Carbon Monoxide Dehydrogenase from *Methanosarcina vannielii*: Purification and Properties. *J. Bacteriol.*, in press, 1987.

Other Publications from the Group:

Ching, W.-M.: Characterization of Selenium-Containing $tRNA^{Glu}$ from *Clostridium sticklandii*. *Arch. Biochem. Biophys.* 244: 137-146, 1986.

Sliwkowski, M.X.: Selenoprotein A of the Clostridial Glycine Reductase Complex: Purification and Amino Acid Sequence of the Selenocysteine-Containing Peptide. *Biochemistry*, in press, 1987.

Wittwer, A.J. and Ching, W.-M.: Selenium-Containing $tRNA^{Glu}$ from *Escherichia coli*: Purification, Codon Specificity, and Translational Activity, in press, 1987.

Miscellaneous:

Lecturer, Institut für Genetik und Microbiologie, University of München, August, 1986.

Invited Lecturer at the Annual Meeting of The Korean Biochemical Society, Chinju City, Korea, October, 1986. (Also presented three lectures at universities in Seoul, Korea).

Invited Speaker - Selenium Mini-Symposium, FASEB Meeting, Washington, DC, April, 1987.

Invited Speaker - 12th International Workshop on tRNAs, Umeå, Sweden, July, 1987.

Organizer of One-Day Biochemistry Session at the 5th International Conference on the Chemistry of Selenium and Tellurium, Oak Ridge Tennessee, August, 1987.

Recipient of the Rose Award (ASBC) - Presented Lecture at the ASBC Meeting in Philadelphia, PA, June, 1987.

Editor-in-Chief, BioFactors, 1987 - Indefinite.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00206-28 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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)


| | | | |
|---|---|---|---|
| PI: | Lin Tsai | Research Chemist | LB, NHLBI |
| Others: | Si-Yu Xu | Visiting Fellow | LB, NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.3 | 2.0 | 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.)


A method for the synthesis of 5-carboxymethylaminomethyl-2-seleno- and 5-carboxymethylaminomethyl-2-thiouracil was developed. This method involved the condensation of selenourea with the enolate of ethyl α-formyl-N-carboxymethylaminopropionate which was generated _in situ_ from ethyl N-carboxymethylaminopropionate.





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

Project Description:

Objectives:

The selenium-modified nucleoside in bacterial tRNAs was established to be 5-methylaminomethyl-2-selenouridine (I, X = Se, R = $CH_2NHCH_3$). The corresponding sulfur-analog (I, X = S, R = $CH_2NHCH_3$) was already known to be present in *E. coli* tRNAs. Recently, 5-carboxymethylaminomethyl-2-thiouridine (I, X = S, R = $CH_2NHCH_2CO_2H$) was recognized as a modified nucleoside in

(I)

*Bacillus subtilis* tRNAs. Therefore, it is of great interest to synthesize the corresponding selenium analog (I, X = Se, R = $CH_2NHCH_2CO_2H$) so as to study its properties and to facilitate the identification of as yet unknown selenium-modified nucleosides that are present in tRNAs of some microorganisms.

Major Findings:

An approach to the synthesis of 5-substituted 2-selenouracil is to condense selenourea with an open-chain intermediate so that the resulting pyrimidine would carry the desired substituent in the 5-position (eq. 1).

(II) + $NH_2$–C(=Se)–$NH_2$ → (III) (1)

This approach is especially expeditious for synthesis of selenopyrimidines because the element of selenium is introduced into the molecule at a late stage of the synthesis thus avoiding unnecessary exposure of the selenium compound to any drastic conditions that might cause extensive decomposition. Accordingly, the crucial stage of the synthesis of the desired pyrimidine derivative was the preparation of the appropriate enolate (II). For this purpose, the amino-esters (IV, a-e) were prepared.

$$\overset{\alpha}{C_2H_5OOCCH_2}CH_2-\underset{\underset{R'}{|}}{N}-\overset{\alpha'}{CH_2}COR''$$

(IV)

| | R' | R'' |
|---|---|---|
| a: | H | OH |
| b: | CHO | OH |
| c: | $COOC_2H_5$ | OH |
| d: | $(C_6H_5)_3C$ | $OC_2H_5$ |
| e: | 9-phenylfluoren-9-yl (structure, labelled φ) | $OC_2H_5$ |

These compounds were designed so that in the presence of a basic reagent and ethyl formate, C-formylation would take place preferentially at α-position, thus producing the enolate (II, R = $CH_2NR'COR''$). The basis for this design was two-fold: (I) The protons (α) adjacent to the ester function would be more acidic than those (α') adjacent to the carboxy group, e.g. (IV, a, b, and c); (II) the bulky group (R') at the N would give rise to steric hindrance more to α' than α-position, e.g. (IV, a and e). Compounds (IV, a-e) were prepared as follows:

$$C_2H_5OOC\text{CH=CH}_2 + NH_2CH_2COR \longrightarrow C_2H_5OOC\text{CH}_2\text{CH}_2NH\text{CH}_2COR''$$

$$\xrightarrow[(Y = Cl \text{ or } Br)]{R'-Y} C_2H_5OOC\text{CH}_2\text{CH}_2\underset{\underset{R'}{|}}{N}\text{CH}_2COR''$$

(IV)

Reactions of (IV) with ethyl formate using various bases were conducted. Compounds (IV, d and e), unfortunately, underwent cleavage at the N-R' group followed by extensive decomposition. Compounds (IV, a, b, and c) did yield the enolate (II) to various extent as indicated by the UV absorption spectrum. So far, the most promising result was the use of sodium hydride as the basic reagent and ethyl formate as reactant as well as solvent. This condition had the advantage of eliminating the necessity of protecting the NH function, since the excess ethyl formate provided N- and C-formylation in the process.

Preliminary experiments indicated that the desired seleno- and thio-pyrimidines could be synthesized albeit in rather low yield as outlined in Scheme I.

Scheme I

The spectral properties of compounds (V, a and b) and (VI,a and b) were consistent with the assigned structures. Moreover, compound (V, a) gave a mass spectrum that revealed the expected isotopic distribution of a single Se-atom in the molecule.

Proposed Course:

Improvement of the present method and exploration of other methodology for the synthesis of 5-substituted 2-selenouracils will be pursued so as to produce well-characterized seleno-compounds for chemical and biochemical studies.

Publications:

DeMoll, E. and Tsai, L.: Conversion of Purines to Xanthine in Methanococcus vannielii. Arch. Biochem. Biophys. 250: 440-445, 1986.

DeMoll, E. and Tsai, L.: Utilization of Purines or Pyrimidines as Sole Nitrogen Source by Methanococcus vannielii. J. Bacteriol. 167: 681-684, 1986.

Wittwer, A.J. and Tsai, L.: 5-Methylaminomethyl-2-Selenouridine, A Novel Selenonucleoside in Escherichia coli tRNA. In Combs, G.F., Jr., Spallholz, J.E., Levander, O.A., and Oldfield, J.E. (eds.): Selenium in Biology and Medicine, Part A, 1987, pp. 222-229.

DeMoll, E., Grahame, D.A., Harnley, J.H., Tsai, L., and Stadtman, T.C.: Carbon Monoxide Dehydrogenase from Methanococcus vannielii. Purification and Properties. J. Bacteriol. 169, in press, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00211-14 LB

PERIOD COVERED
October 1, 1986 to September 30, 1987

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: 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 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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| 1.9 | 0.6 | 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.)


The Fenton reagent (hydrogen peroxide plus Fe(II) or Fe(III)) catalyzes nearly quantitative conversion of amino acids to ammonia and a mixture of aliphatic aldehydes and carboxylic acids containing one less carbon atom. The oxidation is stimulated by bicarbonate ion and additionally by either EDTA, EGTA, o-phenanthroline, Desferal, Ferrozine, DETAPAC, NTA, citrate, or ADP when they are present at concentrations below that required to chelate all of the iron present. With excess chelator, amino acid oxidation occurs only after a lag which corresponds to the time required to reduce (by oxidation) the concentration of chelator to a level slightly below that needed to chelate all of the iron. The results suggest that amino acid oxidation requires the participation of both an iron-chelate and either unchelated iron or a second type of iron complex, possibly one involving bicarbonate ion and/or the the amino acid. Low sensitivity of amino acid peroxidation to various radical scavengers indicates that oxygen radicals are either not involved or are generated _in situ_ by peroxidation of an iron-chelate-amino acid complex. The formation of such a complex was established in spectrophotometric studies showing that Fe(II), ferrozine and an amino acid react to form first a ternary complex which subsequently decomposes to yield the typical (ferrozine)3-Fe(II) complex. The amino acid-Fe(II)-ferrozine-complex but not the (ferrozine)3-Fe(II) complex is readily oxidized by hydrogen peroxide.


75.

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

Project Description:

Objectives: In the presence of Fe(III) and $O_2$, several mixed-function oxidation (MFO) systems have been shown to catalyze the oxidative inactivation of enzymes. Such oxidation is likely involved in the "marking" of enzymes for proteolytic degradation, in the accumulation of altered forms of enzymes during aging, and in the killing of bacteria by neutrophils. The available evidence suggests that $H_2O_2$ and Fe(II) produced by the MFO systems interact at metal binding sites on the target enzymes to form highly reactive oxygen species (OH•, perferryl ion, singlet oxygen, etc.) which attack side chains of amino acid residues at or near the metal binding sites. Among other changes, these amino acid residues are converted to carbonyl derivatives.

A detailed investigation of amino acid oxidation by the Fenton reagent [$H_2O_2$ + Fe(III) or Fe(II)] was undertaken with the hope that it would help to elucidate the mechanism of protein oxidation by MFO systems.

Major Findings:

Reactions 1-6 were found to account for 90-100% of all the peroxide consumed during the oxidation of amino acids by the Fenton reagent.

$$RCH\overset{+}{H}H_3COO^- + H_2O_2 \dashrightarrow RCHO + HCO_3^- + \overset{+}{N}H_4 \quad (1)$$

$$RCH\overset{+}{N}COO^- + H_2O_2 \dashrightarrow RCOCOO^- + \overset{+}{N}_4 + H_2O \quad (2)$$

$$RCH\overset{+}{N}H_3COO^- + 2H_2O_2 \dashrightarrow RCOO^- + \overset{+}{N}H_4 + CO_2 + 2H_2O \quad (3)$$

$$RCH\overset{+}{N}H_3COO^- + 2H_2O_2 \dashrightarrow RCHNON + CO_2 + 3H_2O \quad (4)$$

$$RCHO + H_2O_2 \dashrightarrow RCOO^- + H^+ + H_2O \quad (5)$$

$$2H_2O_2 \dashrightarrow 2H_2O + O_2 \quad (6)$$

Reactions 1 and 2 were monitored by direct measurement of the carbonyl groups by reaction with 2,4-dinitrophenylhydrazine. Reactions 3 and 4 were monitored continuously by monometric measurements of the $CO_2$ produced when the reaction was carried out in bicarbonate-$CO_2$ buffer, in a Warburg apparatus, and when the oxygen produced in reaction 6 was absorbed by oxsorbant present in the side arm of the Warburg flask.

Our previous studies on amino acid oxidation by the Fenton reaction demonstrated: (a) that oxidation requires the presence of bicarbonate ion; (b) oxidation is stimulated by a variety of chelating agents when they are present at concentrations below that required to chelate all of the iron

present, but that all chelating agents, except ADP, completely inhibit amino acid oxidation at concentrations only 1.1-1.2 times that required to sequester all of the iron present; (c) that the oxidation is relatively insensitive to inhibition by radical scavengers.

We now find that with excess chelator, amino acid oxidation does occur eventaully but only after a lag, which increases with the amount of excess chelator, and which corresponds to the time required to decompose (by iron-dependent peroxidation) the amount of chelator in excess of the amount of iron present. It thus appears that amino acid oxidation requires the participation of at least two forms of iron, namely; an iron chelate and also either a small amount of free iron or of a second iron complex, possibly one involving interaction with $HCO_3^-$ or the amino acid.

The insensitivity of amino acid oxidation to radical scavengers indicates either that free radicals are not involved or that the oxidation involves peroxidation of an amino acid-Fe(II)-chelate complex to yield active oxygen which reacts in situ with the amino acid moiety of the complex. The latter interpretation is supported by spectrophotometric studies with Ferrozine. Ferrozine reacts with Fe(II) to form a tight complex $(\text{Ferrozine})_3\cdot\text{Fe(II)}$ which has an absorption maximum at 560 nm ($\varepsilon$ = 27.9 $mM^{-1}$ $cm^{-1}$). This complex is highly resistant to oxidation by $O_2$ or $H_2O_2$. However, when Fe(II) is added to a mixture containing an amino acid and Ferrozine, a ternary complex, $(\text{Ferrozine})_n\cdot\text{Fe(II)}\cdot$amino acid, is formed which has an absorption maximum at 630 nm ($\varepsilon$ = 15.17 $mM^{-1}$ $cm^{-1}$). These ternary complexes decompose to yield the $(\text{Ferrozine})_3\cdot\text{Fe(II)}$ complex with rates dependent upon both the concentration and kind of amino acid involved. In contrast to the $(\text{Ferrozine}_3\cdot\text{Fe(II)}$ complex, the amino acid$\cdot$Fe(II)$\cdot(\text{Ferrozine})_n$ complexes are readily oxidized by $H_2O_2$.

Reaction with Proteins. In view of the above results, the effects of bicarbonate ion and chelating agents on the in vitro oxidative inactivation of E. coli glutamine synthetase (GS) by some mixed-function oxidation system was investigated. The inactivation of GS catalyzed by a MFO sytem comprised of $O_2$, Fe(III) and ascorbate was stimulated by bicarbonate ion and by substoichiometric amounts of iron-chelators, and was inhibited by greater than stoichiometric amounts of iron chelators. A similar, but less pronounced effect of chelating agents on the inactivation of GS by a MFO system comprised of xanthine oxidase, Fe(III), hypoxanthine and oxygen was observed. However, bicarbonate ion had little or no effect on this system.

Proposed Course:

Amino acids react with bicarbonate to yield carbamino derivatives which form stable salts with divalent cations. As shown below, this raises the possibility that iron salts of carbamino derivatives may be implicated in the stimulation of amino acid peroxidation.

$$\mathrm{RCH\overset{+}{N}H_3COO^- + HCO_3^-} \xrightarrow{\mathrm{Fe(II)}} \begin{array}{l} \mathrm{R{-}CH{-}C(=O){-}O} \\ \quad\;\; | \qquad\qquad\quad \mathrm{Fe} \\ \quad \mathrm{NH{-}C(=O){-}O} \end{array}$$

These salts might react further with chelating agents to form adducts with altered redox potentials and thereby affect the ability of the complex to undergo peroxidation and therewith the generation of OH•, which would preferentially attack the amino acid moiety of the complex. Such a mechanism could explain the stimulatory effects of bicarbonate ion and chelating agents and also account for the lack of sensitivity of amino acid oxidation to radical scavengers. The potential role of carbamino derivatives in the oxidation of amino acid will be investigated.

Publications:

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, Vol. 17, 3rd ed., New York, Academic Press, 1986, pp. 21-42.

Ahn, B.-W., Rhee, S. G., and Stadtman, E. R.: Use of fluorescein hydrazide and fluorescein thiosemicarbazide reagents for the fluorometirc determination of protein carbonyl groups and for the detection of oxidized protein on polyacrylamide gels. Anal. Biochem. 161: 245-257, 1987.

Starke-Reed, P. E., Oliver, C. N., and Stadtman, E. R.: Modification of hepatic proteins in rats exposed to high oxygen concentration. FASEB J. 1, 1987, in press.

Oliver, C. N., Levine, R. L., and Stadtman, E. R.: A role of mixed-function oxidation reactions in the accumulation of altered enzyme forms during aging. J. Am. Geriatric Soc., 1987, in press.

Oliver, C. N., Ahn, B.-W., Moerman, E. J., Goldstein, S., and Stadtman, E. R.: Age-related changes in oxidized proteins. J. Biol. Chem. 262: 5488-5491, 1987.

Chock, P. B., Jurgensen, S. R., Rhee, S. G., Stadtman, E. R., and Vandenheede, J. R.: The role of cyclic cascades in metabolic regulation. In Chock, P. B., Tsou, L., and Huang, C. Y. (Eds.): Dynamics of Soluble and Immobilized Enzymes, Amsterdam, Springer-Verlag, in press, 1987.

Chock, P. B., Rhee, S. G., and Stadtman, E. R.: Regulation of glutamine synthetase in E. coli. In Hervé, G. (Ed.): Allosteric Enzymes, 1987, in press.

Shacter, E., Stadtman, E. R., Jurgensen, S. R., and Chock, P. B.: The role of cAMP in cyclic cascade regulation. Meth. Enzymol., in press, 1987.



81.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00212-16 LB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Regulation of Glutamine Synthetase 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 | LB, NHLBI |
| Others: | Edward DeMoll | Staff Fellow | LB, NHLBI |

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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| 1.5 | 1.2 | 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 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. *GlnF* codes for a specific sigma factor, whereas the product of *glnL* appears 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.

It was previously reported that addition of a group of D-amino acids and glycine to *Escherichia coli*, during logarithmic growth in a mineral salts medium containing excess ammonium nitrogen, could effect an increase in the synthesis of glutamine synthetase (GS) comparable to that observed upon ammonia exhaustion. It It was reasoned, therefore, that these amino acids might participate, either directly or indirectly, in the generation of the specific metabolic signal for the nitrogen control response. Subsequent studies reveal that the ultimate recipient of the amino nitrogen of these amino acids is L-serine via an increased serine hydroxymethyltransferase (SHMT) activity in the direction of serine synthesis. No increase in the synthesis of GS could be evoked by addition of the D-amino acids to strains containing mutations in *glyA*, the structural gene for SHMT. Moreover, in the *glyA* mutants, synthesis of glutamine synthetase was not increased in response to limiting ammonia nitrogen by growth in glucose-mineral salts-glutamine medium. The investigation of the mechanism by which the D-amino acids and glycine exert their regulatory effect has uncovered a functional relationship involving *glyA*, *glnB*, and *purL*.





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

Project Description:

Objectives:

(1) To determine the physiological effectors of nitrogen control.

a. Elucidate the mechanism of the D-amino acid provocation of the positive response (1).

b. Identify other components involved in the positive activation process.

(2) 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. Construct glnB-lac fusion strains to study regulation of the $P_{II}$ protein.

b. Resolve genetic inconsistencies with respect to the location of the structural gene for adenylyltransferase (AT).

c. Locate the site of a unique constitutive type mutation, which suppresses glnD, and clarify its regulatory role.

Methods Employed:

(1) The physiological effectors of nitrogen control.

a. Studies on the mechanism of the D-amino acid provocation of the positive response.

Hydrolysates of total cell protein from $^{15}NH_4Cl$-cultured Escherichia coli K12 were examined by mass spectrometry. Addition of D-glutamate; D-thr + D-Lys; or a mixture of D-glutamate, D-lysine, D-threonine, and glycine of natural abundance nitrogen during exponential growth in medium containing excess $^{15}N$ ammonium nitrogen resulted in a pattern of incorporation reflecting increased serine biosynthesis (Table 1).

It appeared likely that serine hydroxymethyltransferase might be responsible for the distribution of $^{14}N$ from the D-amino acids into serine, although the conversion of these amino acids to glycine is not as yet understood. For instance, it is not known whether either threonine dehydrogenase or threonine dehydratase, enzymes which degrade threonine to glycine, can utilize the D-isomer as substrate. The relative increase in serine synthesis from added D-amino acids may also include indirect effects which favor an alternative catabolic route which has yet to be elucidated.

Table 1. Atom % excess $^{14}N$ in amino acids of total protein hydrolysate following addition of amino acids of natural abundance $^{14}N$.[b]

| Amino acid | Additions[b] | | | | | |
|---|---|---|---|---|---|---|
| | Expt 1 | | Expt 2 | | Expt 3 | Expt 4 |
| | 1 h | 2 h | 1 h | 2 h | 1 h | 1 h |
| glu | <0.6 | 3.0 | <0.3 | 4.1 | <0.3 | 20.1 |
| phe | <0.3 | <0.3 | <0.3 | 1.5 | <0.3 | 12.5 |
| asp | <0.3 | 1.5 | <0.3 | 2.1 | <0.3 | 12.8 |
| pro | <0.3 | 1.0 | <0.3 | 2.0 | <0.3 | 12.6 |
| leu | <0.3 | 1.2 | <0.3 | 2.5 | <0.3 | 12.4 |
| ile | <0.3 | 0.4 | <0.3 | 2.4 | <0.3 | 12.2 |
| val | <0.3 | 1.2 | <0.3 | 2.1 | <0.3 | 13.0 |
| ser | 0.8 | 2.6 | 10.3 | 17.0 | 5.3 | 10.8 |
| thr | <0.3 | 0.8 | 7.2 | 7.9 | 8.3 | 16.4 |
| gly | 0.6 | 1.1 | 18.0 | 26.6 | <0.3 | 11.5 |
| ala | <0.3 | 0.4 | 0.9 | 3.4 | <0.3 | 12.2 |

[a]Atom % excess $^{14}N$ values have an average error of ± 0.3.

[b]Expt 1: D-glu; Expt 2: D-glu, D-thr, D-lys, gly; Extp 3: D-thr, D-lys; Expt 4: L-glu, L-thr, L-lys. Amino acids @ 10 mM each were added to E. coli K12 strain N99 growing exponentially in a glycerol-mineral salts medium (2) containing 100 mM $^{15}NH_4Cl$ (ICN, Irvine, CA). Growth was allowed to continue for 1 or 2 hours, as indicated, after which time cells were collected for mass spectrometric measurment of protein amino acids. Cells were harvested at Klett 100 (660 filter) in each case.

The possibility also exists that a racemization of threonine and/or lysine may by facilitated by SHMT, as has recently been shown for another pyridoxal phosphate enzyme (2). This possibility is consistent with the determination that SHMT also has threonine aldolase activity (3). Studies to elucidate the catabolic routes of D-glu, D-thr, and D-lys to glycine are still in progress. However, the data demonstrate that the ultimate recipient of the natural abundance nitrogen, supplied via the addition of these D-amino acids to cells growing in $^{15}NH_4Cl$, is L-serine.

The inhibition of serine degradation by SHMT observed in the presence of D-glu, D-thr, D-lys, and gly *in vitro* (Fig. 1) is consistent with increased activity in the direction of serine synthesis which is observed following the addition of these amino acids to cells growing in excess ammonium nitrogen (Table 1).

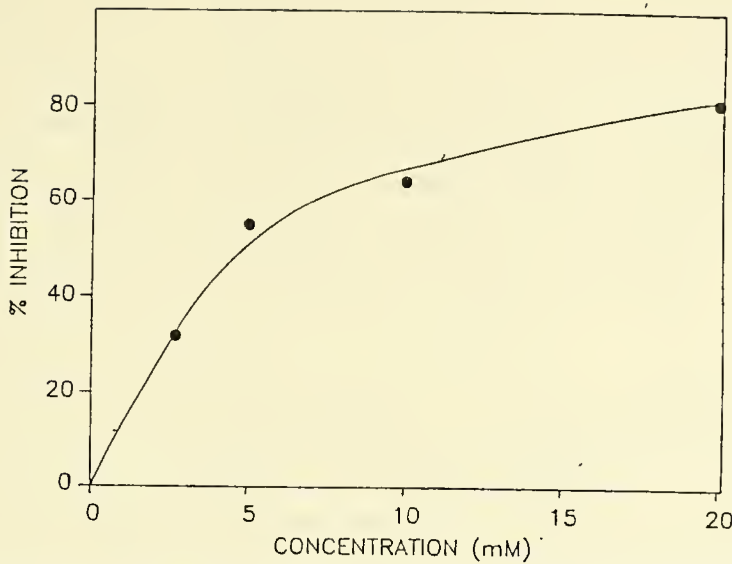


Fig. 1. Inhibition of SHMT activity by D-amino acid mix (conc. = ea. D-glu, D-thr, D-lys, gly) determined with crude extracts in the serine degradative assay (4).

Addition of D- or L-serine failed to effect an increase in the level of GS indicating that the serine product of SHMT is not the putative signal per se. However, a role for SHMT in the generation of the signal is supported by results obtained with strains containing mutations in glyA, the structural gene for SHMT.

Mutants lacking SHMT activity are unable to increase the synthesis of GS in response to D-amino acid addition (Table 2). The relationship between SHMT activity and synthesis of GS can also be observed during ammonia-limited growth. Under these conditions also, no increase in GS synthesis could be observed in glyA mutants (Table 2).

Table 2. Changes in GS level with conditions of nitrogen excess and nitrogen limitation

| Strain[d] | Relevant genotype | GS units/mg N excess[a] | Increase in GS units/mg[f] N limitation[b] | Increase in GS units/mg[f] N excess + D-amino acids[c] |
|---|---|---|---|---|
| GS245 | glyAΔ | 0.32 | < 0 | < 0 |
| AT2457 | glyA | 0.35 | 0.10 | 0.06 |
| N99 | prototroph[e] | 0.40 | 1.40 | 0.64 |

Cells were grown in a glycerol-minteral salts medium with [a]100 mM $NH_4Cl$ or [b]10 mM glutamine as nitrogen source.

[c]D-lysine, D-threonine, D-glutamate, and glycine were added, at 10 mM each, to cells growing in 100 mM $NH_4Cl$ and cultures were harvested after 60'.

[d]GS245: pheA, $thi^{-1}$, ara, $str^r$, Δlac, ΔglyA (G. V. Stauffer). AT2457: $thi^{-1}$, relA, $\lambda^-$, spoT, glyA6 (CGSC). N99: $F^-$, galK, $sm^r$, $\lambda^-$ (NIH collection).

[e]All of the nutrients required for growth of auxotrophs were added to the control. None interferred with D-amino acid effect.

[f]GS assayed by the method of Stadtman et al. (5).



f5.

An increased serine synthesis via SHMT would be likely to perturb the composition of the C-1 donor pool of tetrahydrofolates which might in turn be expected to influence the relative concentrations of certain purine intermediates. However, elimination of the *de novo* purine biosynthetic pathway has no effect on the D-amino acid response (Table 3). This result eliminates the possibility that a purine biosynthetic intermediate, *per se*, could be the putative metabolic signal. Mutants for other amidotransferases did not demonstrate an altered response to D-amino acid addition. Because only certain of the *purL* mutants examined exhibit an altered response (Table 3), an explanation for these findings might best be sought at the genetic level (see 2b below).

(2) Interactions between elements of the modification cascade and genetic regulatory elements.

a. Genetic mapping of *glnB* and construction of *glnB*-lac fusion strains.

The *glnB* gene, which codes for the $P_{II}$ protein of the GS modification cascade, has been cloned from a plasmid bearing the *glyA* locus (6). Subsequent 3-factor crosses (7) have located *glnB* between *purL* and *glyA*. A cotransduction frequency of 82% for *glnB* and *purL* was observed when $pur^+$ was selected. A cotransduction frequency of 78% between *glnB* and *glyA* was observed when $gly^+$ was selected. These data corroborate the proximity of *purL* and *glyA* indicated by the cotransduction frequency of 78% observed when $pur^+$ was selected and 57% when $gly^+$ was selected.

A *glnD*::Tn*10* strain was isolated and phage grown on this mutant were used to transduce *glnD*::Tn*10* into MC4100, a lac deletion strain suitable for selection of lac fusions. By means of λ*placMul* and λpMu507 infection, $lac^+$ fusions resulting in glutamine independent growth were isolated. Because glutamine independent suppressors of *glnD* can be *glnE*, *glnB*, *glnC* or unique constitutive *glnD* su types (see 2c), the purified clones were first assayed for GS to eliminate the *glnE* class. Next, in order to demonstrate that constitutivity and $lac^+$ cotransduce at the 100% frequency which would establish identity, a strain containing a lacΔ was transduced to $lac^+$ using phage prepared from each constitutive isolate. No $lac^+$ fusions to any of the *gln* constitutive loci have as yet been obtained. In the course of producing lac fusions, it was discovered that growth on lactose minimal plates will permit the growth of *glnD* mutants, further complicating *in vivo* selection.

Table 3. Response of relevant biosynthetic mutants to D-amino acid addition

| Strain | Relevant genotype[b] | Reaction affected | Response[c] |
|---|---|---|---|
| MB902 | *purF*::Tn*10* | PRPP-glutamine amidotransferase | + |
| MB904 | *purL*::Tn*10* | PR-formylglycineamidine synthetase | - |
| PA3306 | *purL* | PR-formylglycineamidine synthetase | +d |
| MB905 | *purC*::Tn*10* | PR-aminoimidazole-succinocarboxamide synthetase | + |
| CB101 | *gltB* | glutamate synthase | + |
| MB901 | *car*::Tn*10* | carbamyl phosphate synthetase | + |
| JK24 | *crp* Δ | absence of cAMP binding protein | + |
| GS245 | *glyA* Δ | serine hydroxymethyltransferase | - |
| AT2457 | *glyA* | serine hydroxymethyltransferase | - |

[b]None of the nutrients required for growth of nonisogenic strains showed any interference with the D-amino acid effect when added to the wild type controls.
[c]A positive response, +, indicates the occurrence of an increase in the specific activity of GS equivalent to that of the wild type control, measured at 60' following addition of the D-amino acid mixture to cells growing in glycerol-mineral salts-ammonium medium. No change in GS specific activity is indicated by -.
[d]Increased to 50% of wild type.

b. Unique constitutive type suppressors of glnD.

Another high-level constitutive for GS was isolated as a suppressor of glnD$^-$ and was subsequently removed to a glnD$^+$ background. This isolate, designated K3, can be phenotypically distinguished from glnB by several criteria. Unlike the glnB$^-$ strain, it fails to utilize L-glutamate or arginine as N source, but is extremely sensitive to the analogue, γ-glutamylhydrazide. The high level of GS activity demonstrated by this isolate can be suppressed by an F' comprised of the 13-16.7 minute segment of the *E. coli* chromosome. Crude extracts of this strain contain $P_{II}$ protein which can be uridylylated according to the method of Son and Rhee (6). The amount of $P_{II}$ protein produced by this strain has not been quantitated. Therefore, the possibility remains that K3 specifies a regulatory locus for glnB. The K3 mutation also maps between purL and glyA. In this case, the cotransduction frequency for GS constitutivity observed is 97% with glyA (gly$^+$ selection) and 100% with purL (pur$^+$ selection). In reciprocal crosses, 100% cotransduction frequency between purL::Tn10 and K3 was also observed (tetracycline resistance selected).

Proposed Course:

(1) Identify the physiological effector of nitrogen control which acts at the genetic level.

a. Further investigation of the regulatory consequence of an increased serine biosynthesis via SHMT should ultimately yeild the identity of the specific signal for the increase in GS synthesis which is observed when the D-amino acids are added to cells growing in the presence of excess ammonium nitrogen.

b. Identify additional components as in (2c).

(2) Interactions between elements of the modification cascade and genetic regulatory elements.

a. The process of selecting glnB-lac fusions continues. In addition, lac fusions will be prepared by cassette mutagenesis of the glnB plasmid *in vitro* and will be reinserted into the chromosome. After verification of the correct glnB location, the constructed strain will then be used to study regulation of glnB by assaying B-galactosidase.

b. Determine the specific genetic lesion of the unique constitutive glnD su and clarify its regulatory function with respect to nitrogen control. Studies will include examination of gene x deletions. Gene x is a gene of unknown function which is a component of the divergently transcribed glyA operon (8) and, like K3, is located to the purL side of glyA. Unlike glnB and glyA, gene x is transcribed in a counterclockwise direction according to the convention used to describe transcription of the *E. coli* chromosome (G.V. Stauffer, personal communication).

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7. Berberich, M.A.: Studies on the functional relationship between *purL*, *glnB*, *glyA* and glutamine synthetase regulation in *Escherichia coli* K12. Abstr. *Annu. Meet. Am. Soc. Microbiol.*, p. 165, 1987.

8. Plamann, M.D. and Stauffer: Characterization of the *Escherichia coli* gene for serine hydroxymethyltransferase. *Gene* 22: 9-18, 1983.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00224-10 LB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Calcium-regulated Protein Kinases and Phosphatases

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

| | | | |
|---|---|---|---|
| PI: | Charles Y. Huang | Research Chemist | LB, NHLBI |
| Others: | Aile Zhang | Visiting Fellow | LB, NHLBI |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.2 | 2.0 | 0.2 |

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.)


(1) The calmodulin-dependent protein phosphatase is activatable by Ni(II) in the absence of calmodulin provided some Ca(II) is present. The mechanism of activation is consistent with the random binding of 2 Ni(II) ions which leads to a conformational rearrangement. Experimental and theoretical considerations seem to rule out the existence of an activated intermediate that is reversible to the inactive state on removal of Ni(II). The time-dependent binding of 2 Ni(II) ions to the phosphatase has been confirmed by direct binding studies using radioactive Ni. Phosphorylation of the phosphatase by protein kinase C is further verified by using the catalytic subunit of protein kinase C, the M kinase.

(2) A Ca(II)-inhibited 56,000 MW phosphatase has been purified to >80% purity. Survey of various chromatographic fractions of bovine brain reveals the presence of several Ca(II)-sensitive protein phosphatases whose MW range from 20,000 to 200,000.

(3) Anomalous stoichiometry of protein-ligand and protein-protein interactions as determined by the continuous variation method is discussed for 2 cases: positive cooperativity and ordered reconstitution of unidentical enzyme subunits. It is shown that apparent stoichiometry greater than the true value may be observed at intermediate levels of total protein and ligand. These anomalous stoichiometries should provide clues to the mode of interaction of the components involved.

(4) An improved procedure for the calculation of King-Altman patterns for kinetic reaction schemes has been developed. In this method, each scheme is broken down into 2 diagrams. In one diagram, a selected branch is deleted, while in the other diagram the selected branch must always be present. In the latter diagram, the selected branch is compressed into a point. The sub-diagramming procedure can be repeated as many times as necessary until the resultant diagrams can all be easily calculated.

Project Description

Objectives:

(1) To study the regulatory and catalytic mechanism of enzymes mediated by $Ca^{2+}$, calmodulin, and phosphorylation-dephosphorylation.

(2) To develop or improve methods and theories applicable to enzyme research.

Major Findings:

(1) Mechanistic and structural studies on calmodulin-dependent protein phosphatase. The calmodulin (CaM)-dependent protein phosphatase (CPP) is a $Zn^{2+}$- and $Fe^{2+}$-containing enzyme which requires an additional divalent metal ion such as $Ni^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Mg^{2+}$, etc. for structural stability and catalytic activity. CPP, as isolated, exists in both phosphorylated and dephosphorylated forms, though the functional significance of this covalent modification is not yet understood. Additional studies on these findings are described below:

(a) In the absence of CaM, CPP is activatable by $Ni^{2+}$ provided some $Ca^{2+}$ is present. The time course of $Ni^{2+}$ activation exhibits an initial lag which, unlike that observed in the presence of CaM, does not follow first-order reaction kinetics. When lag times were plotted against reciprocal $Ni^{2+}$ concentrations, a nonlinear (concave downward) relationship was evident. Thus, the activation mechanism was proposed by us to involve the random binding of two $Ni^{2+}$ ions.

A different mechanism has been proposed by Pallen and Wang:

$$E + N \underset{}{\overset{K_1}{\rightleftarrows}} EN \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E^*N \overset{k_3}{\longrightarrow} E^{**}N \qquad (1)$$
$$E^*N \xrightarrow{k_a} P \qquad E^{**}N \xrightarrow{k_b} P$$

where E = CPP, N = $Ni^{2+}$, P = product, and the asterisks denote the activated enzyme forms. Their proposed mechanism was based solely on the observation that on addition of EDTA to chelate $Ni^{2+}$ during activation, there was an immediate substantial decrease of CPP activity. The decrease was taken as evidence for the E*N intermediate which could be reverted to the inactive state by removal of $Ni^{2+}$.

Experimental and theoretical reexamination of this problem argues against the mechanmism of Pallen and Wang:

(i) Their experiment was performed with p-nitrophenylphosphate (PNPP) as substrate in 50 mM Tris buffer, pH 7.0. Since the pK of Tris is around 8, chelation of 1 mM $Ni^{2+}$ by 1 mM EDTA would lower the pH of the Tris buffer to 6.7 owing to the generation of 2 mM proton. This change in pH appears to be the main reason for the observed decrease in PNPP phosphatase activity because both CPP activity and the absorbance of p-nitrophenol are reduced. A similar experiment performed by us at pH 7.6 using 200 mM Hepes buffer showed little change in CPP activity upon addition of EDTA.

(ii) The sudden decrease of CPP activity is more consistent with a sudden change in pH. It cannot be explained by rapid equilibration between EN and E*N since it would reduce the scheme in Eqn. 1 to a simple first-order reaction. A slow conversion between EN and E*N, on the other hand, should give rise to a slow, first-order decay of CPP activity when Ni is removed.

(iii) Theoretically, Eqn. 1 predicts that the lag time, $\tau$, is linearly proportional to the reciprocal $Ni^{2+}$ concentration:

$$\tau = (1/k_3)\{(1-k_a/k_b) + [(k_{-2}+k_3)/k_2](1+K_1/N)\} \qquad (2)$$

This prediction is contrary to our experimental data.

(b) Binding studies were carried out by incubating CPP with 5 mM $^{63}Ni^{2+}$ in the absence or presence of CaM at pH 7.6, 30°C. $Ca^{2+}$ (0.1 mM) was present in all experiments to ensure that the $\beta$ subunit of CPP was not deficient in $Ca^{2+}$. EDTA was added to a final concentration of 5 mM to terminate the binding after 2 or 60 minutes of incubation. The samples were exhaustively dialyzed against buffer containing 0.1 mM EDTA and 0.1 mM $Ca^{2+}$ and then counted to determine the amount of irreversibly bound $Ni^{2+}$. The presence of CaM did not seem to significantly affect the outcome even though it did influence the mechanism of $Ni^{2+}$ activation. The amount of bound $Ni^{2+}$ was about 0.8 mol per mol enzyme after 2 minutes of incubation and about 1.8 mol per mol enzyme after 60 minutes. The results support the concept that two mol of $Ni^{2+}$ per mol of CPP are irreversibly bound and the amount bound is dependent on the length of incubation time.

(c) Phosphorylation of CPP by protein kinase C previously reported by us has been verified by another laboratory (Tung, 1986, Biochem. Biophys. Res. Commun. 138, 783-788). We have further demonstrated this phosphorylation by employing the catalytic fragment of C kinase -- the M kinase. It was found that freshly prepared CPP served as good substrate for both C kinase and cAMP-dependent kinase; whereas CPP stored at -70°C for several months could not be phosphorylated by either kinase.

(2) Purification and characterization of $Ca^{2+}$-sensitive phosphatases from bovine brain.

(a) Using PNPP as substrate, we have purified a $Ca^{2+}$-inhibited phosphatase to >80% purity. The procedure includes homogenation and centrifugation, $(NH_4)_2SO_4$ cuts, DEAE-cellulose chromatography, S-200 gel filtration, CaM-sepharose column, and Mono-Q column (FPLC system). Gradient gel electrophoresis of the final sample revealed one major active band of $M_r \sim 56,000$ and two minor bands.

(b) Assay of various protein fractions from the DEAE-cellulose and S-200 column steps described above using histone 2B as substrate revealed several $Ca^{2+}$-sensitive phosphatases whose molecular weights range from 20,000 to 200,000. The 200,000 $M_r$ phosphatase does not hydrolyze PNPP.

(3) Anomalous stoichiometry of protein-ligand and protein-protein interactions as determined by the continuous variation method -- the Job plot. In the continuous variation method, the total molar concentration, $C_o$, of two interacting components is held constant while their mol fractions, X and Y are

continuously varied. A measurable parameter that is linearly proportional to the complex(es) formed, $\Sigma$, is plotted against the mole fractions, and the intersection point of the two limiting slopes, $d\Sigma/dX)_{X\to o}$ and $d\Sigma/dY)_{Y\to o}$, are determined. The binding stoichiometry, n, is calculated from the ratio of the mole fractions of these two components at the intersection point of these slopes, $Y_i/X_i$. Normally, the observed n value varies between one and n as $C_o$ increases relative to the magnitude of the dissociation constant(s) involved. Recent developments indicate that under certain circumstances the apparent stoichiometry can become greater than the true n value. These anomalous situations point to the need of performing the Job plot experiments at a wide range of $C_o$ in order to obtain the correct n value and to gain knowledge of the mode of binding. Two special cases will be discussed.

(a) Cooperative binding: consider a simple Adair model in which the two binding sites of a protein are initially equivalent but binding of the first ligand affects the affinity of the second site. The ratio of the mole fractions at the intersection point, R, is given by

$$R = \frac{Y_i}{X_i} = \frac{K_1K_2 + (K_1+2K_2)C_o+2C_o^2}{K_1K_2 + 2K_2C_o + C_o^2} \qquad (3)$$

Although R approaches the correct value of 2 when $C_o \gg K_1$, $K_2$ and reaches 1 when $C_o \ll K_1$, $K_2$, it can be seen from Eqn. 3 that R may become greater than 2 at intermediate levels of $C_o$ if $K_1 > K_2$, i.e., when the cooperative phenomenon is a positive one. Differentiation of Eqn. 3 with respect to $C_o$ yields the condition for the maximum value of R at $(dR/dC_o) = 0$ where

$$C_o = K_1[K_2 + K_2(K_1-K_2)]/(K_1-K_2) \qquad (4)$$

Let $K_1 = 10K_2$, then $C_o = 5K_2$ and $R = 2.67 > 2$. This example shows that for the positive cooperative binding case, the apparent n value may rise from unity to a value greater than the true n and then back to n as $C_o$ increases.

(b) Another system that may generate similar situations is the reconstitution of enzyme with unidentical subunits. For instance, the formation of a tetrameric $\alpha_2\beta_2$ enzyme may take place according to the following reactions:

$$\alpha+\beta \overset{K_1}{\rightleftharpoons} \alpha\beta$$

$$\alpha\beta+\alpha \overset{K_2}{\rightleftharpoons} \alpha_2\beta$$

$$\alpha_2\beta+\beta \overset{K_3}{\rightleftharpoons} \alpha_2\beta_2$$

Let X and Y be the mole fractions of $\beta$ and $\alpha$, respectively, it can be shown that

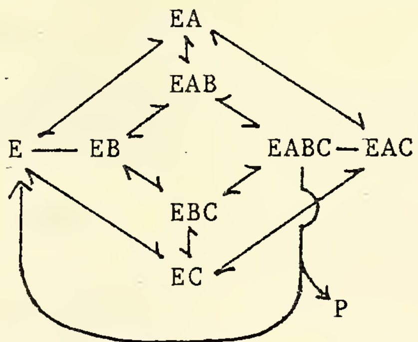
$$R = \frac{Y_i}{X_i} \simeq \frac{K_1K_2 + (2K_1+K_2)C_o + 2C_o^2}{K_1K_2 + K_2C_o + {}_K C_o^2} \qquad (5)$$

Eqn. 5 is analogous to Eqn. 3; it predicts that R will rise from unity to a value greater than unity and then approach unity again as $C_0$ is increased. This phenomenon reflects the formation of $\alpha_2\beta$ in the reconstitution process. If the $\alpha\beta_2$ species is also formed, any deviation of R from unity may not be detected due to the symmetry of the reaction pathways.

It should be noted that anomalous n values can also occur because of the method employed for determination of complex formation. If both $\alpha_2\beta$ and $\alpha\beta_2$ forms exist but only $\alpha_2\beta$ is catalytically active, detection of the extent of reconstitution by enzymic activity will lead to abnormal n values. Thus, ideally Job plots should be done with different methods of measuring the complexation. It should also be noted that, unlike the Hill coefficient, the apparent n value of a negatively cooperative system will not become smaller than unity (see Eqn. 3 when $K_1 < K_2$; $n < 1$ may occur, however, if one reverses the assignment of X and Y).

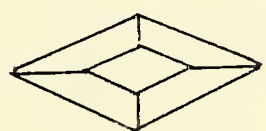
(4) <u>Improved graphical method for calculating King-Altman patterns for complex kinetic reactions.</u> The graphical method for calculating King-Altman patterns, originally developed by Huang, permits rapid computation of the total valid patterns in most instances. An improved procedure has been devised for highly complex reactions which may involve three-dimensional schemes and for calculation of the exact number of patterns of individual enzyme species in a scheme containing irreversible steps. Basically, the method breaks the parent scheme into two diagrams by selecting a reference branch such that in one diagram the reference branch is deleted, while in the other it is obligatory. The new approach can be best illustrated by appropriate examples:

(a) Three-dimensional reaction scheme: consider the kinetic scheme for an enzyme with three substrates A, B, C binding in a random fashion:

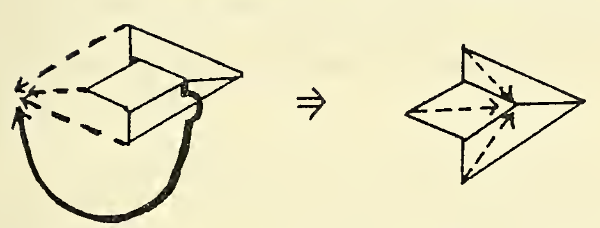



The above three-dimensional scheme can be split into two diagrams using the EABC→E step as the reference branch:

(i) The EABC→E branch is deleted. The resultant diagram is planar and can be shown to contain 384 valid patterns.

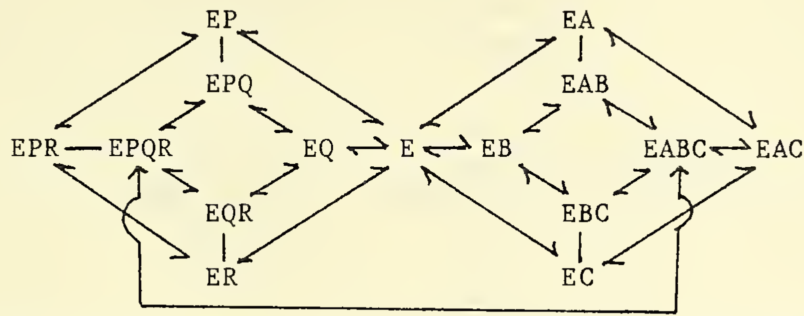


(ii) The EABC→E branch (heavy-lined) must be present in all valid patterns. Graphically, it amounts to compressing the E and EABC nodes into a single node; i.e., the branches leading into E (dashed arrows) are to be directed into EABC, and the EABC→E branch disappears due to the compression. The redrawn diagram can be calculated to contain 320 valid patterns.



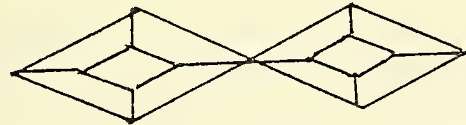
Combining the patterns obtained in (i) and (ii) above, we arrive at a total number of 704 for the three-dimensional reaction scheme.

(b) Complex three-dimensional schemes containing two subschemes linked by a common node:



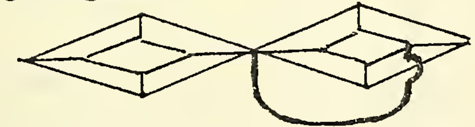

The reversible reaction scheme of the random binding of three substrates, A, B, C, and random release of products P, Q, R, shown above, contains two subschemes joined by E. For special cases like this, the parent scheme can be broken down into three diagrams by choosing the EABC⇌EPQR branch as the references:

(i) The reference branch is deleted:



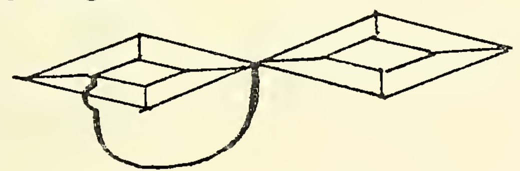
384 X 384 patterns

(ii) The reference branch is compressed with respect to the substrate-binding diagram:



384 X 320 patterns

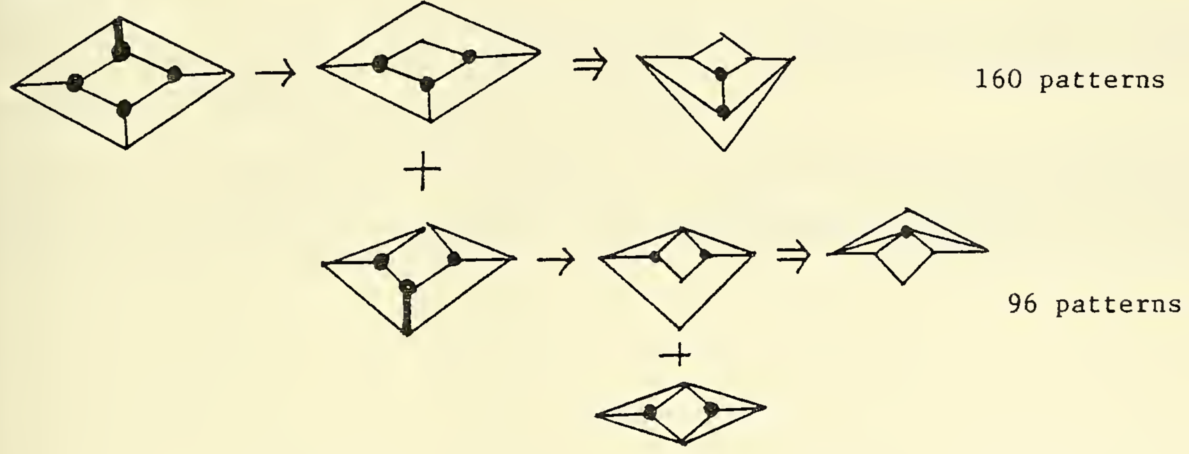
(iii) The reference branch is compressed with respect to the product-binding diagram:



320 X 384 patterns

The total valid patterns = 384 (384 + 320 + 320) = 393216. Note that the number of patterns for each of the above sub-diagrams has been calculated in part (a). It should also be noted that the matrix approach for calculating valid patterns for this reaction scheme is manually infeasible because of the ~ 8.7 X $10^{10}$ steps involved.

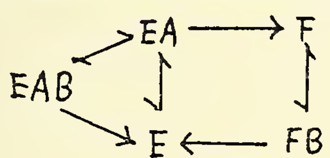
(c) Reduction of a reaction scheme to less complicated diagrams to facilitate calculations: The procedure described above can be repeated as many times as necessary. For instance, calculation for one of the diagrams in part (a) is tedious as indicated by the four intersection points present (solid circles). The diagram can be converted to simpler ones by using successive reference branches (marked by heavy lines; hollow arrows denote redrawing to a simpler form).



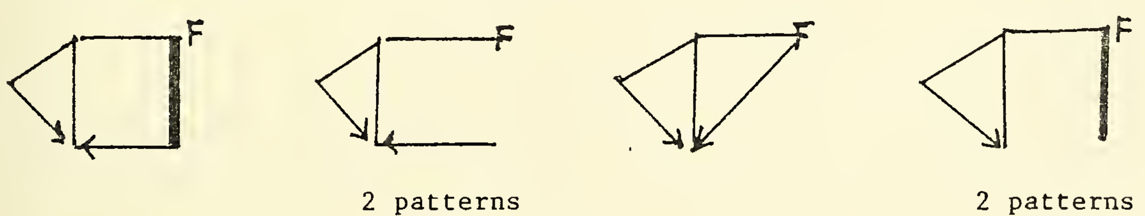

128 patterns

Total patterns = 160 + 96 + 128 = 384.

(d) Reaction schemes containing irreversible steps: Consider the following scheme for a bisubstrate reaction with dual ordered and ping-pong pathways,




which contains three irreversible steps. In choosing the reference branch, one must use the reversible ones. Also, as has been described in previous reports, unidirectional branches leading to a node whose King-Altman patterns are to be determined are considered as reversible; whereas branches leading away from that node are omitted. For example, the number of patterns for the F node can be obtained fromt he following operations.



Proposed Course:

(1) Role of $Ca^{2+}$ and phosphorylation in the regulation of calmodulin-dependent protein phosphatase. Identification of kinase and phosphatase involved in the phosphorylation-dephosphorylation of this enzyme.

(2) Purification and characterization of various forms of $Ca^{2+}$-sensitive phoshpatases.

(3) Continuation of development of theory and method applicable to enzyme research.

Publications:

Jayaram, M. N., Cooney, D. A., and Huang, C. Y.: Interaction between L-aspartic acid and L-asparaginase from *Escherichia coli*: Binding and inhibition studies. *J. Enzymol. Inhibition* 1: 151-161, 1986.

Huang, C. Y., Lanciotti, M., and Zhang, A.: Mechanism of activation of calmodulin-dependent phosphatase by divalent metal ions. In Chock, P. B., Huang, C. Y., Tsou, C. L., and Wang, J. H. (Eds.): *Enzyme Dynamics and Regulation*, Amsterdam, Springer-Verlag, in press, 1987.



6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00225-10 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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)*

| | | | |
|---|---|---|---|
| PI: | Rodney L. Levine | Senior Investigator | LB, NHLBI |
| Others: | Michel Chevalier | Visiting Fellow | LB, NHLBI |
| | Isabel Climent | NIH Special Volunteer | LB, NHLBI |
| | Anke Lenz | NIH Special Volunteer | LB, NHLBI |

COOPERATING UNITS *(if any)* Laboratory of Cellular Metabolism, NHLBI; Instituto de Investigaciones Citólogicas de la Caja de Ahorros de Valencia, Valencia, Spain; Department of Enzymology, Gesselschaft für Strahlen- und Umweltforschung (GSF), Munich, West Germany.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.5 | 2.7 | 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.)*


Chronic and acute oxygen toxicity is implicated in a growing list of pathologic processes. The three major biopolymers are targets of oxygen toxicity, nucleic acids, lipids, and proteins. Many proteins are subject to covalent modification by mixed function oxidation. In general, oxidatively modified enzymes lose catalytic activity and become susceptible to proteolytic degradation. Degradation is mediated by specific proteases which recognize the modified proteins but do not degrade the native forms. Experiments with bacterial glutamine synthetase have established that the susceptibility to proteolysis correlates with the oxidation of the second of two histidine residues and also with increased absorbance at 320 nm.

The lung is susceptible to oxidative attack by molecular oxygen and by air pollutants. A sensitive assay to detect oxidatively modified proteins in human pulmonary lavage specimens has been developed. This assay will allow detection of oxidized proteins which may occur in certain diseases or as a result of exposure to air pollutants.

Oxidative modification of proteins may also have physiological roles; for example, in controlling the metabolic switch from anaerobic to aerobic metabolism in Klebsiella. When these cells are switched to an oxygen atmosphere, glycerol dehydrogenase undergoes rapid inactivation and proteolytic degradation. The process requires synthesis of RNA and protein; it can also be triggered by low concentration of hydrogen peroxide, without protein synthesis.




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

Project Description:

Objectives:

Glutamine synthetase and many other bacterial and eukaryotic enzymes are now known to be susceptible to mixed function oxidation, mediated by a variety of enzymic and nonenzymic systems. The physiologic and pathologic functions remain to be determined, although the modification is known to render glutamine synthetase and other proteins susceptible to proteolytic degradation. The objectives of this project include determination of the chemical and structural changes induced by oxidation; purification and characterization of the systems which catalyze the modification and subsequent proteolysis; and understanding the controls which may regulate the modification and proteolysis of specific proteins.

Major Findings:

* Last year's report summarized detailed studies on chemical and physical changes in glutamine synthetase upon exposure to a model oxidizing system for varying times. Those studies were expanded and confirmed using independent techniques. The major conclusions are: (1) Early in the time course the enzyme is inactivated due to the oxidation of histidine-269, which causes loss of an essential cation-binding site. (2) Additional exposure generates multiple forms of more highly oxidized protein which vary in isoelectric point. (3) The process does not cause gross denaturation of the protein; sedimentation velocity is actually unchanged. (4) A second histidine residue is oxidized with longer exposure to the system and loss of this residue correlates well with susceptibility to proteolytic degradation. (5) Loss of the second histidine also correlates with increased absorbance at 320 nm, providing a convenient indirect assay for proteolytic susceptibility.

* We are continuing studies of glycerol dehydrogenase in *Klebsiella aerogenes*. When abruptly switched from an anaerobic to an aerobic environment, glycerol dehydrogenase activity is rapidly lost. Experiments this year demonstrated: (1) The inactivated enzyme is degraded very rapidly. Thus far, no intermediate products have been found on Western blots. (2) Inactivation is reversibly blocked by inhibitors of protein synthesis or of RNA transcription. (3) Inactivation and proteolysis can be triggered anaerobically by exposure to hydrogen peroxide (in the presence of glycerol). The process does not require protein synthesis. This finding and observation (2) suggest that exposure to oxygen induces synthesis of a protein(s) which generates the hydrogen peroxide required for inactivation. It also follows that the protease which degrades the modified glycerol dehydrogenase is already present in anaerobically growing cells.

* The lung is the organ which is exposed to the highest partial pressures of oxygen and also exposed to oxidizing agents from air pollution and cigarette smoke. Patients, especially prematurely-born neonates, develop pulmonary damage due to the toxic effects of treatment with oxygen. The oxidative modification of lung proteins can be studied on lavage samples from

animals and humans. We therefore developed a sensitive assay for oxidative modification of proteins in lavage fluid, based on earlier work in the laboratory. This technique will detect a 10% modification of a sample containing 50-100 μg protein. We are now completing a survey of lavage specimens from humans with a variety of pulmonary diseases.

* Many studies would be facilitated by a sensitive, stable "label" for oxidatively modified proteins. Building on previous work in the laboratory, we are attempting to develop such compounds. In general, these are carbonyl reagents which react with the carbonyl groups of oxidized proteins. Several hydrazines react well, but systematic study failed to find a method to stabilize the resultant hydrazones. Current attempts now focus on utilizing fluorescent amines to form a Schiff base followed by reduction to the stable amine.

* Glutamine synthetase from E. coli is subject to covalent modification by adenylylation or mixed-function oxidation. In collaboration with Dr. J. Moss we have shown that it is also ADP-ribosylated by an ADP-ribosyl transferase from erythrocytes. Peptide maps revealed that arginine-172 is the modified residue. This residue forms part of a central loop which extends into the core of the dodecameric structure; this region is also susceptible to cleavage by a variety of proteases which inactivate the enzyme. Similarly, ADP-ribosylation causes a 90% loss in $Mg^{2+}$-dependent biosynthetic activity and a 70% loss in $Mn^{2+}$-dependent γ-glutamyl transferase activity. Both adenylylated and unadenylylated glutamine synthetase as well as mixed-function oxidized enzyme are substrates for the ADP-ribosyl transferase.

Significance to Biomedical Research:

Chronic and acute oxygen toxicity is implicated in a growing list of pathologic processes which include arthritis, the aging process, carcinogenesis, bronchopulmonary dysplasia and adult respiratory distress syndrome, retinopathy of prematurity, and reperfusion-mediated ischemic damage. Oxidative modification of proteins has been demonstrated in some of these disorders and likely occurs in all of them. Studies noted above demonstrate that oxidatively modified proteins are rendered susceptible to proteolytic degradation by proteases which attack modified but not native proteins. Oxidative modification might also function physiologically; for examples, in controlling the switch from anaerobic to aerobic metabolism, in host defense mechanisms, in limitation of the inflammatory repsonse, and in some mechanisms of protein turnover.

Proposed Course:

Studies will continue on the physicochemical changes in glutamine synthetase upon oxidative modification. The second modified histidine residue must be identified as well as the residue causing the UV spectral change. In collaboration with Dr. J. Villafranca, we plan to study the proteolytic susceptibility of site-specific mutants of glutamine synthetase. Other studies are directed at assessing the role of oxidative modifications of proteins in pathologic and physiologic processes. These include further investigations

of the "aerobic switch" in Klebsiella and new studies of oxidative modification of purified enzyme from aged animals. Finally, significant effort will be directed towards study of the oxidative modification of proteins from the human immunodeficiency virus. The goal is the development of compounds which specifically oxidize those proteins.

Publications:

Roseman, J.E. and Levine, R.L.: Purification of a protease from *Escherichia coli* with specificity for oxidized glutamine synthetase. *J. Biol. Chem.* 262: 2101-2110, 1987.

Oliver, C.N., Levine, R.L., and Stadtman, E.R.: A role of mixed-function oxidation reactions in the accumulation of altered enzyme forms during aging. *J. Amer. Geriatric Soc.* 34, in press, 1987.

Rivett, A.J. and Levine, R.L.: Enhanced proteolytic susceptibility of oxidized proteins. *Biochem. Soc. Trans.*, in press, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00241-08 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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)

PI: Todd M. Martensen NIH Special Volunteer LB, NHLBI

COOPERATING UNITS (if any)

Department of Biological Chemistry, Johns Hopkins University, Baltimore, MD
Laboratory of Vision Research, NEI

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| 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.)


Immunodecoration of proteins containing Tyr-P on electroblots is possible by incubating the electroblot of SDS gels with sheep antibodies which bind Tyr-P. The region of the blot with bound antibodies is detected with affinity purified anti•sheep IgG conjugated with horseradish peroxidase. The procedure was tested with authentic proteins containing Tyr-P or Ser-P residues and appears to be specific. This has been used to identify calpactin in lens protein extracts and protein substrates of several growth factor receptor tyrosine kinases.

Immunoadsorption of phosphotyrosyl proteins (PTP) has been accomplished by immobilization of anti-phosphotyrosine (a-YP) antibodies to protein-A Sepharose or covalent linkage by CNBr activated Sepharose. These preparations allow semi-quantitative adsorption of tyrosine phosphorylated epidermal growth factor receptor and the insulin receptor.




PHS 6040 (Rev. 1/84) 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:

Protein interconversion reactions such as phosphorylation-dephosphorylation can often be studied with alternative substrates advantangeously (Martensen, 1985). A model phosphotyrosine protein was used as a substrate for calcineurin, a putative protein phosphatase of broad substrate specificity. The velocity with the macromolecular substrates was $Ca^{2+}$ and calmodulin insensitive and $\sim 10^{-3}$ that for nitrophenyl phosphate suggesting that specificity for protein tyrosine phosphate residues is not apparent; the activity resembles that of nonspecific phosphomonoesterases (Kincaid, Martensen, and Vaughn, 1986).

The effect of orthovanadate, an inhibitor of certain protein tyrosine phosphate phosphatases *in vitro*, was found to amplify cellular phosphotyrosine levels using *in vivo* incubations which indicate that orthovanadate can be used to characterize cellular protein phosphotyrosine phosphatases and amplify certain substrates of protein tyrosine kinases (Gentleman, Reid, and Martensen, 1986).

Antibodies to Tyr-P have been used to decorate PTP separated by SDS PAGE on Western blots. The technique of utilizing these antibodies along with antiprotein antibodies on 2-D gel blots allows the identification that a known protein is tyrosine phosphorylated *in vitro* or *in vivo*. Coincident immunodecoration by anti-calpactin antibody and a-YP antibody identified a lens protein as phosphocalpactin (Russell, Zelenka, Martensen, and Reid, 1987).

Treatment of 3T3-L1 adipocytes with insulin, insulin-like growth factors 1 and 2, epidermal growth factor, and platelet-derived growth factor resulted in characteristic PTP patterns seen on Western blots. With insulin and

insulin-like growth factor a 160 kDA protein becomes labeled in a time and insulin dependent manner. This protein's phosphorylation appears to be dependent on insulin suggesting it is a specific substrate for these two tyrosine kinases (Madoff, Martensen, and Lane, submitted).

Immunoadsorption of phosphotyrosyl proteins (PTP) has been accomplished by two methods. The first involves the incubation of soluble or detergent solubilized PTP with antiphosphotyrosine (a-YP) antibodies from sheep, rabbit anti-sheep IgG ($Ab^2$) antibodies, and protein-A Agarose. The complex of PTP and (a-YP) is bound to the $Ab^2$ protein-A complex. This aggregate has been shown to bind the epidermal growth factor receptor kinase after the protein is synthesized but before the protein is glycosylated, a requirement for binding epidermal growth factor (Sleiker, Martensen, and Lane, 1986).

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 of uncharacterized proteins. Understanding the role of these substrates in cellular activities and regulation of the kinase activity will be useful to understanding a variety of human diseases at the molecular level.

Proposed Course:

Immunoabsorption and desorption of proteins containing Tyr-P can be used to detect kinase or other activities of Tyr-P modified proteins in cells. Specific attention will be focused on GTP binding or phospholipase C modulation of immunoadsorbed material found in cells. By this method the function of normal or transformed cell phosphotyrosyl proteins can be investigated. Evidence for ligation of biological molecules to phosphorylated protein tyrosine residues will be investigated by pronase digestion of radioactive protein which after denaturation in SDS fail to bind to immobilized a-YP antibodies yet yield Tyr-P after partial acid hydrolysis.

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, (Publisher) 1985, Vol. 27, 171-181.

Slieker, L.J., Martensen, T.M., and Lane, M.D.: Synthesis of Epidermal Growth Factor Receptor in Human A431 Cells: GlycosylationDependent Acquisition of Ligand Binding Activity Occurs PostTranslationally in the Endoplasmic Reticulum. J. Biol. Chem. 261: 15233-15241, 1986.

Kincaid, R.L., Martensen, T.M., and Vaughan, M.: Modulation of Calcineurin Phosphotyrosyl Protein Phosphatase Activity by Calmodulin and Protease Treatment. Biochem. Biophys. Res. Commun. 140: 320-328, 1986.

Gentleman, S., Reid, T.W., and Martensen, T.M.: Vanadate Stimulation of Phosphotyrosine Protein Levels in Quiescent Nakano Mouse Lens Cells. Exptl. Eye Res. 44: 587-594, 1986.

Lane, M.D., Slieker, L.J., Olson, T.S., and Martensen, T.M.: Posttranslational Acquisition of Ligand Binding and Tyrosine Kinase-Domain Function by the Epidermal Growth Factor and Insulin Receptors. In J. Brockaert (Ed.): Symposium on Purification, Biosynthesis, and Regulation of Membrane Receptors. INSERM Publication, Marcel Dekker Publisher, in press, 1986.

Russell, P., Zelenka, P., Martensen, T., and Reid, T.W.: Identification of the EDTA-Extractable Protein in Lens as Calpactin I. Curr. Eye Res. 6: 533-538, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00255-04 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Carbon and Nitrogen Metabolism in Methanococcus vannielii.

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

PI: L. Edward DeMoll, III Staff Fellow LB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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☐ (a1) Minors

☐ (a2) Interviews

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

I further characterized the purine degrading pathway of Methanococcus vannielii. The first three steps are identical to those described for clostridia. However, the final enzyme in the clostridial purine metabolic pathway, glycine reductase, was not found in M. vannielii. I purified xanthine dehydrogenase from M. vannielii. The native enzyme has a molecular weight of 230,000 with subunits of molecular weights of approximately 85,000 and 31,000.

Additionally, I purified carbon monoxide dehydrogenase from M. vannielii, and determined it to be a protein of molecular weight 220,000 with subunits of molecular weights of 89,000 and 21,000. I discovered that the previously unseen CODH-catalyzed reduction of carbon dioxide could be driven by the reduced form of a low potential viologen. Additionally, I found that CODH could catalyze C-14 exchange between carbon dioxide and carbon monoxide.

Project Description:

The biochemistry and physiology of *Methanococcus vannielii*. (1) Nitrogen metabolism in *Methanococcus vannielii*. (2) Acetate synthesis in *Methanococcus vannielii*.

Objectives: (1) To determine the mechanisms of purine and pyrimidine utilization by *Methanococcus vannielii*. (2) To determine the mechanism of acetate synthesis from $CO_2$.

Major Findings:

1. I previously characterized the first two steps in purine metabolism, (1) the hydrolytic cleavage of xanthine to 4(5)-ureidoimidazole-5(4)carboxylic acid (I) and (2) the hydrolytic cleavage of (I) to 4(5)-aminoimidazole carboxylic acid (II). In this past year I have characterized the third step, the decarboxylation of (II) to 4-aminoimidazole. I have also determined that there is no detectable glycine reductase activity in extracts of *M. vannielii*. Therefore, the purinolytic pathway is different from any other previously described.

   I have purified xanthine dehydrogenase from *M. vannielii* to near homogeneity. In the purification I used various types of chromatography: DEAE-cellulose, Sepharose-6B gel filtration, phenyl-Sepharose, and hydroxylapatite. An ammonium sulfate precipitation was also employed. As determined by gradient SDS-gel electrophoresis, the enzyme has two subunits of molecular weights 85,000 and 31,000. From gradient gel electrophoresis I determined that the native enzyme has a molecular weight of approximately 230,000. This suggests an $\alpha_2\beta_2$ structure for the intact enzyme. Only about 3% of the enzyme activity was recovered in the purification. Appreciable loss of activity was observed at each step.

2. We have implicated carbon monoxide dehydrogenase (CODH) as having a role in acetate synthesis in *M. vannielii*. I have purified carbon monoxide dehydrogenase 580-fold from crude extracts of *M. vannielii*. The purification consisted of an acetone precipitation step, and DEAE-cellulose, phenyl-Sepharose, hydroxylapatite chromatographic steps. The native enzyme has an $\alpha_2\beta_2$ structure, with subunits of molecular weights 89,000 and 21,000 as determined by SDS-gradient gel electrophoresis. Based in part on other work in this laboratory, we conclude that this enzyme is identical to one isolated from acetate-grown *Methanosarcina barkeri*. Since *M. vannielii* is incapable of growth with acetate as sole carbon source, this implies that in methanogens, the same enzyme is involved in both acetate synthesis and breakdown.

   In order to examine the mechanism of acetate synthesis it will be necessary to establish an *in vitro* acetate synthesizing system. CODH has been shown to catalyze the oxidation of carbon monoxide. If the enzyme has a role in acetate synthesis, then it must also catalyze the reduction of $CO_2$ to CO. It is necessary for us to be able to reduce $CO_2$ in this manner



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to fulfill one of the requirements of an _in vitro_ acetate synthesizing system. This reaction has a relatively low redox potential, -540 mV, so we had to come up with a source of electrons with a redox potential low enough to drive the CODH-catalyzed reduction of $CO_2$. We have synthesized 1,1'-trimethylene-2,2'-dimethyl-4,4'-dipyridylium dibromide, a viologen with a redox of -656 mV, and I have shown that it does drive the CODH catalyzed reduction of $CO_2$ to CO. Additionally, I have demonstrated that CODH also catalyzes reaction (1).

$$(1) \quad {}^{14}CO_2 + CO \rightleftharpoons {}^{14}CO + CO_2$$

Proposed course:

1. I will continue to characterize the purine degrading pathway. I have begun to characterize the pyrimidine degrading pathway. I am presently trying to determine the cause of the loss of xanthine dehydrogenase activity, and am initially looking at the possibility that the electron acceptor, 2,3,5-triphenyltetrazolium chloride, which I use in the assay, is not the best. I am preparing to try 2,6-dichlorophenol indophenol.

2. I plan to continue assembling an _in vitro_ acetate synthesizing system. The next step will be to isolate the donor of the methyl group in acetate synthesis.

Significance to Biomedical Research and the Program of the Institute:

The ecological role of methanogens is to remove reducing equivalents from anaerobic environments. Several types of infections are caused by anaerobic bacterial consortia. The methanogens are probably not themselves pathogenic, but their presence is required for growth of the pathogens. Understanding methanogen physiology will make possible another avenue of approach for the treatment or prevention of anaerobic infections.

Publications:

DeMoll, E. and Shive, W.: Assay for Biotin in the Presence of Dethiobiotin with _Lactobacillus plantarum_. _Anal. Biochem._ _158_: 55-58, 1986.

DeMoll, E. and Tsai, L.: Utilization of Purines or Pyrimidines as Sole Nitrogen Source by _Methanococcus vannielii_. _J. Bacteriol._ _167_: 681-684, 1986.

DeMoll, E. and Tsai, L.: Conversion of Purines to Xanthine by _Methanococcus vannielii_. _Arch. Biochem. Biophys._ _250_: 440-445, 1986.

DeMoll, E., Grahame, D.A., Harnly, J.M., Tsai, L., and Stadtman, T.C.: Carbon Monoxide Dehydrogenase from _Methanococcus vannielii_. Purification and Properties. _J. Bacteriol._ _170_, in press, 1987.

DeMoll, E. and Shive, W.: Replacement of Biotin by Dethiobiotin for Growth of _Lactobacillus plantarum_. _Appl. Environ. Microbiol._, in press, 1987.

Berberich, M. A. and DeMoll, E.: Studies on the Functional Relationship between *purL*, *glnB*, *glyA*, and Glutamine Synthesis Regulation in *Escherichia coli* K-12. *J. Bacteriol.*, in press, 1987.



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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00259-03 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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 | LB, NHLBI |
| Others: | P. Boon Chock | Section Chief | LB, NHLBI |
| | Ephrem Tekle | Biological Technician (Summer Student) | LB, NHLBI |

COOPERATING UNITS (if any) T. Y. Tsong, Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland; H. V. Westerhoff, Section on Theoretical Molecular Biology, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, Maryland

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.6 | 1.3 | 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.)


1. We have formulated a theory for understanding the effect of an oscillating or fluctuating membrane potential on the structure and function of membrane protein. In particular, implications concerning the mechanisms of biological signal and transduction have been explored.

2. An apparatus, designed and constructed in conjunction with BEIB, has been developed for the purpose of experimentally testing the above theories. The device allows for the application of an oscillating electric field across a suspension of cell with an amplitude up to ± 150 V/cm (± 1000 V/cm in a different mode) with a very flexible wave shape. Preliminary experiments indicate that indeed there is a non-ohmic response of cell suspensions to an increase in the field amplitude. The frequency dependence can also be determined.




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

Project Description:

Objectives: To develop, and experimentally test, a theory for the effects of oscillating membrane potentials on the dynamics of biological signal and energy transduction.

Major Findings:

Theoretical. Electrical fluctuations that occur spontaneously across biological membranes differ in a number of significant aspects from their homogeneous phase counterparts. While deviations of a thermodynamic parameter from its mean value in bulk solution are typically small ($\sim 1/V^2$) and dissipate on a time scale rapid as compared to most chemical events, excursions of the local membrane potential (caused by, e.g., opening and closing of ion channels) from the average value may be quite large and relax on a time scale determined by a protein conformational transition. Often these fluctuations display effectively coherent behavior, giving rise to macroscopically observable oscillations. For example, the voltage across β-pancreatic cells displays periodic burst activity, depending on the amount of glucose in the perfusion media. The amplitude of these oscillations is 40 mV (i.e., a shift in the transmembrane electric field strength of ≈ 80 kV/cm) with a period on the order of 10 Hz. Similar phenomena have been observed in a wide range of other systems. It must be realized that these local fluctuations, and macroscopic oscillations, are manifestations of energy releasing processes, i.e., the movement of ions down their electrochemical gradients. This energy can be harnessed by many ordinary membrane proteins and enzymes for signal and energy transduction.

It might be thought that chemical reactions in the presence of external, or even energy driven, environmental fluctuations can be described in terms of simple deterministic kinetic equations, where the rate and equilibrium coefficients are those relevant for the average value of the fluctuating parameter. This is the case only if two conditions are met. First, the fluctuation amplitude must be small, such that non-linear effects due to the intrinsic exponential dependence of rate and equilibrium coefficients on thermodynamic parameters are not manifested. Second, the fluctuation dissipation rate (i.e., inverse correlation time) must be great to insure that cross correlations between the external "noise" (or oscillation) and the enforced variation of the concentrations of chemical reactants can be safely neglected. In general, neither of these conditions are met by membrane potential ($\Delta\psi$) fluctuations across biological membranes.

This is an important consideration in light of the fact that the conformational transitions of many transmembrane proteins have a significant electric susceptibility. It is particularly evident in the case of voltage gated channels, but will come into play for any conformational change involving either intramolecular charge transfer or rotation of dipole groups such as α-helices. This clearly includes, but is not limited to, all ion transport ATPases, ion carriers, and electron transfer proteins in the electron transfer chain (ETC).



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We have computationally demonstrated that energy can be transduced from oscillating or fluctuating fields by both direct numerical integration, in which the concentrations of substrate, S, and product, P, were followed and by matrix inversion where S and P were taken to be constant and the accumulation of cyclic flux was followed.

Similar concepts have allowed for the development of a model for cytosolic free energy transduction based solely on protein-protein interactions between the enzyme catalyzing the reactions to be coupled. This presents a significant evolutionary advantage in that proteins can be "engineered" in terms of their interactions with each other and the environment to couple arbitrary enzyme-catalyzed chemical reactions for the purpose of signal and free energy transductions. In particular, membrane proteins must be treated as active, rather than passive, electrical elements in mediating the transfer of energy between metabolic energy stores and ion currents.

Experimental. A device for applying oscillating electric fields (amplitude up to ± 150 V/cm) to cell suspensions has been designed and constructed. We have incorporated the ability to monitor the transient conductance of both a sample and reference cell, taking and storing the output data into a computer for subsequent analysis. By looking at the differential conductivity between a sample and reference mixture (one may be a cell suspension and the other just buffer; or they may both be cell suspensions where in one an inhibitor of a specific membrane protein has been added) allows for the very sensitive detecting of small changes as a function of field frequency or amplitude.

Comparison of the Fourier spectrum of the input periodic potential and the output current signal will allow for detection and analysis of non-linear responses of membrane processes to stimulation. We anticipate that it will be possible to directly determine rate coefficients for a variety of membrane processes, much in the same way that stationary relaxation techniques are used in homogeneous solution. In cell suspensions, there is the added advantage that relatively small input electric perturbations are effectively amplified in (and only in) the region of interest, i.e., across the membrane, due to the low conductance of the bilayer.

Additionally, subsequent to relatively long term (1-5 hr) electrostimulation of a cell suspension, wet chemical techniques can be used to ascertain whether any differences between the sample and an identical control not subject to stimulation have arisen. For example, we will look at $^{32}P$ incorporation into various protein and $^{3}H$-inositol incorporation into the lipid bilayer. Substance transport can also be followed by radiotracer methods.

Significance to Biomedical Research and the Program of the Institute:

Many physiologically important functions rely on periodic stimuli for their regulation. Perhaps the most obvious is the heart itself, which contracts and relaxes about 60 times each minute. It is not at all understood how a biochemical DC stimulus is converted to an AC signal, and finally how

this oscillating signal at the cellular level engenders a macroscopic response. An interesting theoretical observation is that often a critical number of individual "cooperating" cells is needed before a regularly periodic response emerges. By using external perturbation technique to enforce regular oscillation, we may be able to understand, at a biochemical level, how oscillations influence the dynamics of many membrane proteins to elicit the observed physiological effects.

Our studies are also designed to investigate how the physical asymmetry experienced by membrane proteins can be utilized by the system to influence, and thereby regulate, their function. In particular, we are hopeful that understanding of the effects of changes in the membrane potential on membrane proteins can be related to the anomolous membrane potentials often observed in the cells of diseased organisms.

Proposed Course:

At this point, it seems that theory has outpaced experiment. Thus, we propose to devote the major effort in the next year to experimental aspects of the problem. In particular, we will attempt to perfect transient monitoring techniques to obtain kinetic information on membrane protein conformational transition, especially voltage sensitive ion channels in neuroblasts; we will investigate effects of oscillating fields on active lactose transport in *E. coli*, and we will monitor $^{32}P$ incorporation into proteins in neuroblasts. These aspects will be greatly facilitated by the fact that the predictions based on the theories we have formulated and described are quite clear.

Publications:

Tsong, T.Y. and Astumian, R.D.: Absorption and Conversion of Electric Field Energy by Membrane Bound ATPases. *Bioelec. Bioenerg.* 211: 457-476, 1986.

Westerhoff, H.V., Tsong, T.Y., Chock, P.B., Chen, Y., and Astumian, R.D.: How Enzymes Can Capture and Transmit Free Energy Contained in an Oscillating Electric Field. *Proc. Natl. Acad. Sci. U.S.A.* 83: 4734-4738, 1986.

Astumian, R.D., Chock, P.B., Tsong, T.Y., Chen, Y., and Westerhoff, H.V.: Can Free Energy Be Transduced From Electrical Noise? *Proc. Natl. Acad. Sci. U.S.A.* 84: 434-438, 1986.

Astumian, R.D., Chock, P.B., and Tsong, T.Y.: Absorption and Conversion of Energy from Dynamic Electric Fields by Membrane Proteins: Electroconformational Coupling. *Studia Biophysica* 119: 123-130, 1987.

Tsong, T.Y. and Astumian, R.D.: Electro-Conformational Coupling and Membrane Protein Function. *Progress in Biophysics and Molecular Biology*, in press, 1987.

Astumian, R.D., Chock, P.B., Westerhoff, H.V., and Tsong, T.Y.: Energy Transduction by Electroconformational Coupling. In P.B. Chock, C. Huang, L. Tsou, and J.H. Wang (Eds.): Enzyme Dynamics and Regulation. Amsterdam, Springer-Verlag, 1987, in press.

Westerhoff, H.V. and Astumian, R.D.: The Dynamics of Electrostatic Interactions Between Membrane Proteins. In G.R. Welch (Ed.): Towards a Cellular Enzymology. New York, Plenum Press, 1987, in press.

Westerhoff, H.V., Kamp, F., Tsong, T.Y., and Astumian, R.D.: Interactions Between Enzyme Catalysis and Non-Stationary Electric Fields. In M. Blank and E. Findl (Eds.): Mechanistic Approaches to Interactions of Electromagnetic Fields with Living Systems. New York, Plenum Press, 1987, in press.

Tsong, T.Y., Chauvin, F., and Astumian, R.D.: Interaction of Membrane Proteins with Static and Dynamic Electric Fields Via Electroconformational Coupling. In M. Blank and E. Findl (Eds.): Mechanistic Approaches to Interactions of Electromagnetic Fields with Living Systems. New York, Plenum Press, 1987, in press.

Astumian, R.D., Chock, P.B., Chauvin, F., and Tsong, T.Y.: Electroconformational Coupling and the Effects of Static and Dynamic Electric Fields on Membrane Transport. In M. Blank and M. Markov (Eds.): Electromagnetic Fields and Biomembranes. New York, Plenum Press, 1987, in press.

Tsong, T.Y. and Astumian, R.D.: How Transport ATPases Use Electric Field Energy. _Annual Reviews of Physiology_, in press, 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00261-02 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

CO Dehydrogenase and Acetoclastic Methanogenesis in *Methanosarcina barkeri*

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

PI: David A. Grahame NIH Special Volunteer LB, NHLBI

COOPERATING UNITS (if any)

Supported by grant to T.C. Stadtman from Gas Research Institute of Chicago.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| 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.)

Large quantities of purified carbon monoxide dehydrogenase (CODH) have been obtained from acetate-grown cells of *Methanosarcina barkeri* by scaling-up the purification procedure which we developed in 1985. Studies on the pure enzyme demonstrated that it has an $\alpha_2\beta_2$ oligomeric structure composed of subunits with molecular weights of 19,700 and 84,500. Analysis of the metal ion content indicated 1.3 $\pm$ 0.3 (n=4) Ni and 15.6 $\pm$ 5.6 (n=5) Fe per mol $\alpha_2\beta_2$. Dialysis against EDTA did not reduce the content of these metals. The enzyme did not contain Co or Mo. The amino acid compositions of the subunits were determined. The enzyme did not catalyze isotopic exchange between [1-C14]acetyl-CoA and CO. Ferredoxin, FAD, FMN, 2,3,5-triphenyltetrazolium chloride (TTC), methyl viologen, and phenazine methosulfate served as electron acceptors; however, the enzyme did not reduce NAD, NADP, or the 8-hydroxy-5-deazaflavin factor F-420. A higher degree of thermostability was noted in the absence than in the presence of CO. Pure CODH from *Methanococcus vannielii* was also found to be an $\alpha_2\beta_2$ oligomer composed of subunits which were similar in molecular weights to the subunits of *M. barkeri* CODH.

Project Description:

Objectives:

The overall objective of this research is to identify the enzymatic steps involved in the reduction to methane of the methyl group of acetate and certain other one-carbon compounds (e.g., methanol, formate, $CH_3$-SCoM, or $CH_3$-$B_{12}$). Studies on the mechanism of action of the enzymes involved would ultimately provide information which could be used in constructing synthetic or semi-synthetic catalysts for industrial purposes, and would be extremely helpful in the design of methods to improve the efficiency and reliability of bioconversion processes.

In order to accomplish this it is necessary to purify the component enzymes and cofactors and carry out reconstitution of a defined enzymatic, methanogenic system. Only with highly purified preparations can the involvement of a suspected component be conclusively demonstrated. To gain an understanding of the chemical mechanisms involved in methane biosynthesis it is essential to carry out thorough physicochemical characterization of each enzyme in the pathway. For this, too, highly purified enzyme preparations are absolutely essential. Thus, our initial goals are to develop procedures by which the enzymes suspected to be involved can be purified in sufficiently large quantities for biochemical studies of their structure and function. After this has been done (and the enzymes have been well characterized) experiments can be undertaken to determine their exact roles and mechanisms of action in methanogenesis.

Acetate is an important intermediate substrate in the complete anaerobic microbial degradation of biomass yielding methane and carbon dioxide (1-3). Acetic acid serves as a carbon and energy link between acetogenic microorganisms (those producing acetate from anaerobic fermentation of hexoses) and the specific methanogens which degrade it to carbon dioxide and methane. Of special interest are organisms in the genera Methanosarcina and Methanothrix since these are the only methanogens known to be capable of growth on acetate as the sole carbon and energy source (4,5). Fermentation of acetic acid by these bacteria proceeds as follows:

$$CH_3COOH \rightarrow CH_4 + CO_2$$

It has been known since the late 1940's that methane is derived from the methyl group and carbon dioxide from the carboxyl group of acetate (6,7). However, the mechanism of this important reaction is not understood since the enzymes which catalyze it have not been identified.

The involvement of carbon monoxide dehydrogenase (CODH) in acetate degradation has been inferred largely due to its role in acetate biosynthesis by acetogenic members of the Clostridia species which has been demonstrated by Wood et al. (8). In addition, Ferry (9) and Zeikus et al. (10) have independently observed that the levels of CODH are elevated when methanol-grown species of Methanosarcina are subsequently adapted to growth on acetate.

Recent immunological experiments by Zeikus et al. (11) also indicate involvement of CODH in methane formation from acetate. The precise role of this enzyme in acetate catabolism, however, has not been determined.

The specific objectives for 1986 were:

1. Accumulate sufficient quantities of acetate-grown M. barkeri and use these cells to develop a large-scale method for purification of CODH. Obtain quantities of the pure enzyme needed for biochemical and physicochemical characterization.

2. Demonstrate conclusively the oligomeric structure of the enzyme.

3. Carry out quantitative analyses of the metal ion components of CODH, with particular attention to the iron, nickel, and cobalt contents.

4. Determine complete and accurate amino acid compositions of both $\alpha$ and $\beta$ subunits of CODH and gain insight into their redox properties from their contents of cysteine.

5. Conduct tests for isotopic exchange of CO with the carbonyl group of $[1-^{14}C]$acetyl-CoA to establish whether pure CODH from M. barkeri catalyzes an exchange reaction similar to that of CODH from acetogenic clostridia. These experiments are to be conducted in collaboration with Dr. Steve Ragsdale, a Postdoctoral Fellow at Case Western Reserve University, who has carried out this type of analysis on purified CODH from C. thermoaceticum (8) and will be able to compare accurately the results using our pure CODH from M. barkeri.

6. Analyze the thermal stability of CODH in the absence and presence of carbon monoxide to test for substrate protection. Obtain information useful in the interepretation of the physiological significance of carbon monoxide as a susbstrate for CODH.

7. Investigate a number of compounds for reaction with CODH as possible electron acceptors. These studies are aimed at understanding the pathway by which electron equivalents can be transferred to or from CODH by other cellular components. Among the compounds to be tested are: $NAD^+$, $NADP^+$, FAD, FMN, phenazine methosulfate, ferredoxin, and the 8-hydroxy-5-deazaflavin cofactor (factor $F_{420}$) which is unique to methane bacteria.

8. Collaborate with Dr. Edward DeMoll on purification and characterization of CODH from Methanococcus vannielii. Begin preliminary studies to compare the M. vannielii enzyme to CODH from M. barkeri.

9. Submit for publication a paper describing the aggregation behavior, methods developed for purification, and major findings to date on the properties of purified CODH from M. barkeri.

Major Findings:

1. The procedure which we developed for purification of CODH from M. barkeri was successfully scaled-up to provide large amounts of the pure enzyme. The large-scale procedure was quite reproducible and has been carried out three times yielding a total of over 30 mg of the pure enzyme. Large amounts of pure CODH were required for analyses of physicochemical and catalytic properties which we have conducted and will also be needed in future experiments to elucidate the metabolic role of CODH in methanogenesis from acetate.

2. Three independent measures of the subunit stoichiometry have confirmed a 1:1 ratio of $\alpha$ and $\beta$ subunits in CODH. Results from chemical crosslinking experiments and native gradient gel electrophoresis have conclusively demonstrated that the oligomeric structure of CODH is of the type $\alpha_2\beta_2$.

3. Analyses of the metal ion content of pure CODH by plasma emission spectroscopy have determined the content of a number of metal ions of biological importance. The enzyme was found to contain significant amounts of iron (1.5 ± 5.6) and nickel (1.3 ± 0.3 atoms per mol of enzyme). And, analysis following dialysis against EDTA has established that catalytically important metals are tightly bound by the enzyme. The enzyme did not contain molybdenum which distinguishes it from the molybdenum-containing CODH from the aerobic microorganism Pseudomonas carboxydovorans. We have also noted the absence of cobalt which demonstrates that purified CODH is essentially free from contamination by corrinoid proteins. This is important since further experiments to reconstitute an acetate-driven methanogenic consortium of enzymes will probably involve addition of one or more purified corrinoid components.

4. We have conducted accurate and complete amino acid analyses of both CODH subunit polypeptides. Previously, information on the amino acid compositions of the M. barkeri CODH subunits was not available and our analyses have accomplished this. As an upshot, we have gained insight on the subunit location of iron-sulfur centers. Since iron-sulfur centers in proteins coordinate to cysteine, we have paid particular attention to the quantities of this amino acid in each subunit. Analyses revealed that the small ($\alpha$) subunit contains very little cysteine (1.3 residues/mol) and that the majority of cysteine (23.1 residues/mol) is present in the large ($\beta$) subunit. The implications from these findings are that iron-sulfur centers (and, in all likelihood, the enzyme active site(s)) are located on the $\beta$ subunits.

5. Tests for isotopic exchange between carbon monoxide and [$1-^{14}C$]-acetyl CoA were carried out in collaboration with Dr. Steve Ragsdale using clostridial CODH as a model system. It was determined that M. barkeri CODH does not catalyze the same exchange reaction that the clostridial enzyme does. This finding may well be related to a difference in the metabolic roles of these enzymes and suggests that M. barkeri CODH may participate

in acetate conversion to methane and $CO_2$ by a mechanism which is not analogous to the reversal of acetate biosynthesis.

6. Results of thermal stability tests have demonstrated that CODH is exceptionally heat stable. However, carbon monoxide rendered the enzyme more susceptible to thermal inactivation. The thermal destabilization by CO is of interest since it clearly indicates that CO does not function in a typical role of substrate protection. The findings suggest that CO is not a physiologically important substrate for CODH and point to the possibility of testing other compounds for protection against thermal inactivation as a means of identifying the "true" substrate.

7. A number of compounds have been tested as possible electron acceptors with purified CODH. In the presence of CO the enzyme transferred electrons to: Methyl viologen, 2,3,5-triphenyltetrazolium chloride, phenazine methosulfate, FAD, FMN, and ferredoxin, but $NAD^+$, $NADP^+$, and the 8-hydroxy-5-deazaflavin (factor $F_{420}$) were not reduced. The results show that reducing equivalents from CODH do not directly enter the cellular pool of reduced $F_{420}$ and indicate that ferredoxin or flavoproteins (or flavin derivatives) may be involved in CODH-mediated electron transfer processes.

8. In collaboration with Dr. Edward DeMoll we have purified CODH from *Methanococcus vannielii*, which is now the second pure CODH obtained from a methanogen. A similar purification protocol was successfully used and initial studies have revealed a close structural similarity to CODH from *M. barkeri*. Although *M. barkeri* and *M. vannielii* are morphologically, metabolically, and taxanomically substantially different, they are, nevertheless, both methanogens. And, the finding that their CODHs are structurally alike (and different from all other known bacterial CODHs) is of great interest since it suggests a unique metabolic role of CODH in methanogenesis.

9. We have compiled data and prepared a manuscript documenting, in large part, the results from research during 1985-1986. This has been published in *The Journal of Biological Chemistry* and is listed under *Publications*.

Proposed Course:

1. Further investigations are planned on the properties of purified CODH. Several of these are as follows:

   (a) A study of the subunit interactions will be carried out by preparing antibodies specific to the $\alpha$ and $\beta$ subunits. Tests for inhibition of CODH by one or both of the antibodies will provide supporting evidence on the subunit location of the active site. In addition, we will use our antibodies raised against *M. barkeri* CODH to test for cross-reactivity with CODH from *M. vannielii*. This type of experiment will add much to our understanding of the relatedness of these enzymes. The availability of antibodies will also allow us to construct an affinity column consisting of anti-CODH antibodies

immobilized on agarose gel. This column will be used as a means of resolving crude extracts of CODH. Pure CODH will then be added back to test for reconstitution of methanogenesis from acetate and thereby establish its involvement.

(b) Direct information on the subunit location of active site amino acid residues will be obtained by carrying out site-specific labeling experiments with $[^{14}C]$-$CH_3I$ and/or $[2-^{14}C]$-phenylglyoxaldehyde both in the absence and presence of CO ± methyl viologen.

(c) It remains to be established whether or not disulfide groups are present in CODH. To address this question, carboxymethylation with iodoacetic acid (IAA) will be carried out anaerobically on the native and guanidine•HCl denatured enzyme. Effects on the iron-sulfur centers will also be monitored in these experiments.

Ultimately, it will be important to isolate peptides following chemical and/or enzymatic modification of CODH and carry out amino acid sequence analysis of these fragments. This direction of the research will yield benefits not only for an understanding of structural and catalytic properties of the enzyme, but also in the possible design of oligonucleotide probes for study of the control and expression of CODH on the genetic level.

2. Experiments will be conducted to demonstrate methyl group transfer from acetate to reduced cobamide derivatives. Formation of methylcobalamin from methanol and $B_{12}s$ by extracts of <u>M. barkeri</u> has been demonstrated (12) and methanol:5-hydroxybenzimidazoylcobamide methyltransferase has been purified (13,14). Since acetate-grown <u>M. barkeri</u> also possesses corrinoids in large amounts, it is possible that there exists an analogous enzyme, acetate:5-hydroxybenzimidazolylcobamide methyltransferase. Therefore, extracts of acetate-grown <u>M. barkeri</u> will be tested for methyl group transfer by incubation with $[2-^{14}C]$acetate and either $B_{12}s$ or reduced 5-hydroxybenzimidazolylcobamide (this compound would be obtained by purification from <u>M. barkeri</u> according to standard procedures). Analyses will be made for the production of $[^{14}C]$-methylcobamide derivatives. In order to develop an efficient assay system, tests will be conducted to determine whether ATP is required for the reaction and conditions such as pH and cobamide derivative concentration will be explored.

3. When assay conditions have been established, the crude extract will be fractionated in order to purify the enzyme(s) involved in methyl group transfer. It is likely that these experiments will require strictly anaerobic procedures and be conducted entirely within the NIH Anaerobic Laboratory. We will initiate physicochemical characterization of these enzyme(s) following the development of a successful method for their purification.

4. CODH will be tested early on for participation in methyl group transfer to cobamide derivatives. The pure enzyme will be added to test for

stimulation of methyl group transfer in the crude and/or purified methyltransferase preparations. Under the conditions of these experiments, further analyses will be made in order to test for the isotope exchange reaction. These experiments are designed to establish whether CODH is a key enzyme in methanogenesis from acetate and, if so, to elucidate its metabolic role.

5. Since methylcobalamin:HS-CoM methyltransferase (an oxygen-stable enzyme) has been previously isolated (15), combining the purified enzymes needed for methylcobalamin formation from acetate with this enzyme and the purified $CH_3$-S-CoM methylreductase complex should yield a system of defined enzymes and cofactors capable of methane generation from acetate. We will attempt this type of reconstitution experiment following successful purification of the enzymatic components.

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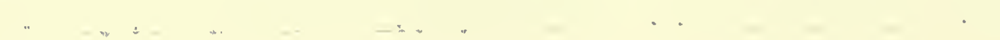
Publications:

Grahame, D.A. and Stadtman, T.C.: Carbon Monoxide Dehydrogenase from Methanosarcina barkeri: Disaggregation, Purification, and Physicochemical Properties of the Enzyme. J. Biol. Chem. 262: 3706-3712, 1987.

DeMoll, E., Grahame, D.A., Harnley, J.H., Tsai, L., and Stadtman, T.C.: Carbon Monoxide Dehydrogenase From Methanococcus vannielii. Purification and Properties. J. Bacteriol. 169, in press, 1987.

Grahame, D.A. and Stadtman, T.C.: In Vitro Methane and Methyl Coenzymes M Formation from Acetate: Evidence that Acetyl-CoA is the Required Intermediate Activated Form of Acetate. Biochem. Biophys. Res. Commun., in press, 1987.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00262-02 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

The Role of Oxidative Modification in Cellular Protein Turnover and Aging

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


| | | | |
|---|---|---|---|
| PI: | Pamela E. Starke-Reed | Staff Fellow | LB, NHLBI |
| Others: | Earl R. Stadtman | Chief | LB, NHLBI |


COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.4 | 1.1 | 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.)*


During normal aging, cellular enzymes accumulate as catalytically inactive or less active forms. The mechanisms regulating these changes, as yet unknown, may involve mixed-function oxidation of critical amino acid residues. In an effort to define the mechanisms responsible for these alterations, we have developed an in vivo model system to compare the protein changes observed during aging with changes mediated by exposure of the animals to an acute oxidative stress. We have examined the levels of oxidized proteins, enzyme inactivation, and protease activity in hepatocytes isolated from rats exposed to 100% oxygen or from rats of various ages. The levels of oxidized protein increase with animal age and also with exposure to 100% oxygen up to 48 hours followed by a sharp decrease between 48 and 54 hours. A decrease in the specific activity of glutamine synthetase and glucose-6-phosphate dehydrogenase was observed under each experimental condition and preceded the loss of immunological cross reactivity indicating the accumulation of inactive proteins. Hepatic protease activity at pH 2.5, 5.5, 7.4, and 8.8 was assayed in each sample. The acid proteases showed little variation under both conditions; however, the alkaline protease activity decreases with age and remains constant during the first 48 hours of oxygen exposure with a significant increase in activity observed between 48 and 54 hours. Alkaline protease activity correlated inversely with accumulation of oxidized protein.

These results invite speculation that the accumulation of oxidized protein and the inactivation of enzymes which occur when animals are exposed to a high concentration of oxygen may reflect an acceleration of the same metabolic changes that lead to the accumulation of oxidized proteins and enzyme inactivation during aging and accelerated aging diseases.




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

Project Description:

Objectives: We are continuing our investigations into the biochemical alterations of cellular enzymes during normal aging and the effects of oxidative stress on this process.

Major Findings:

Previous studies have demonstrated that many cellular enzymes accumulate as catalytically inactive or less active forms during cellular aging. The mechanism(s) whereby these enzymes become altered has yet to be clearly defined. The possibility that some of the age-related alterations involve the oxidation of critical amino acid residues in enzymes is suggested by the demonstration that many of the enzymes which are known to accumulate as inactive forms are highly susceptible to oxidative inactivation by various mixed-function oxidation systems (MFO). A role of such oxidative damage in the turnover of enzymes is suggested by the fact that after their oxidative inactivation enzymes become highly susceptible to degradation by proteases present in both the cytosol and lysosomes. The further observation that substrates of the enzymes protect them from oxidative damage suggests that the regulation of the turnover of an individual enzyme is mediated by variations in the levels of its substrates.

In the continuation of our studies we have examined the levels of oxidatively modified proteins in hepatocytes from young and old rats and from young rats exposed to 100% oxygen. Mixed-function oxidation-mediated oxidative inactivation of proteins leads to the generation of protein carbonyl derivatives which can be detected by reactivity with 2,4-dinitrophenylhydrazine (DNPH) resulting in the formation of a stable protein hydrazone derivative. We examined the levels of protein carbonyl groups in the soluble fraction of hepatocytes isolated from rats exposed to 100% oxygen for 0, 24, 48, or 54 hours and from rats ages 3, 12, 20, or 26 months. The level of oxidized proteins increases with 100% oxygen exposure up to 48 hours and is followed by a sharp decline between 48 and 54 hours. Protein oxidation also increases with age from 3 to 26 months.

Previous studies with glutamine synthetase (GS) *in vitro* have indicated that although MFO-mediated inactivation is associated with the loss of a single histidine in each subunit, further oxidation of the protein leads to increased susceptibility to proteolysis by proteases selective for oxidatively modified protein. In order to ascertain whether specific enzymes were inactivated *in vivo* under these conditions, we determined the specific activity and the levels of immunological cross reacting material of two liver enzymes, glutamine synthetase (GS), and glucose-6-phosphate dehydrogenase (G-6-PD). The specific activity of GS and G-6-PD was decreased significantly during the 54 hours of oxygen exposure and also during aging between 3 and 26 months. Antibody titration of GS and G-6-PD revealed that the amount of antibody required to precipitate 50% of the activity of either enzyme remained constant or increased with time of exposure to 100% oxygen until 48 hours and then

markedly declined. During aging the amount of immunologically reactive protein also decreased more slowly than the loss of catalytic activity. These data indicate that inactive protein accumulates under both experimental systems and together with the protein oxidation results suggests that the loss of catalytic activity correlates with increased protein carbonyl content.

The accumulation of oxidized proteins during oxygen exposure and aging suggests that either the rate of oxidation exceeds the proteolytic capacity or the proteases which selectively degrade the oxidized proteins are inactivated or deficient, or both. Proteolytic activity of extract preparations from hepatocytes of oxygen-treated rats or rats of various ages was determined at four pH values: Two acidic and two basic. The acid protease activity remained relatively constant, but the alkaline protease activity increased with time during the first 48 hours of exposure to 100% oxygen. The rate of protein oxidation exceeds the capacity of the cell to degrade the oxidized protein; however, with further exposure (48 to 54 hours) there is an increase in alkaline protease activity leading to a rapid loss of oxidized protein. From a comparison of the protein carbonyl data it appears that the accumulation of oxidized protein during aging may be caused by a decrease in alkaline protease activity.

These findings suggest that the accumulation of oxidized proteins and the inactivation of enzymes that occur when animals are exposed to 100% oxygen may reflect an acceleration of the same metabolic changes that lead to the accumulation of oxidized protein during aging and accelerated aging diseases.

Findings From Related Experiments:

Studies in other laboratories have indicated that *ad libitum* versus restricted feeding greatly influences the life span in rodents. We have obtained mice from the same litter, one group eating *ad libitum* and the other group having a restricted diet, and compared the effects of the two eating protocols on the levels of oxidatively modified protein in the liver. We did not see any significant differences in the levels of oxidized proteins between the two groups.

Other studies have indicated that exercise also affects the potential life span of rats; therefore, we investigated the possibility of correlation between protein carbonyl content and exercise. We determined the levels of oxidatively modified proteins in the hind leg muscles from trained rats and litter-paired sedentary rats. We found an increase in the level of protein carbonyl groups in the muscle samples from the trained rats compared to the samples from the sedentary rats. After a two-week period of no exercise for either group, we again examined the levels of oxidized proteins in the muscles and found the level of protein carbonyl groups in the trained animals had returned to approximately the same level as that in sedentary rats. We concluded that vigorous exercise increases the oxidative modification of muscle proteins and this increase appears to correlate with a decrease in life span.

Proposed Course:

We will continue to investigate accumulation of altered enzymes during normal aging; specifically examining the involvement of the following age-related changes in this process:

a) an increase in the level of mixed-function oxidation systems;
b) a decrease in the levels of metabolites or enzymes which scavenge activated oxygen species;
c) a decrease in the levels of metabolites that protect enzymes from oxidative damage; and
d) further elucidation of the alkaline proteases selective for oxidized proteins which appear to be inactivated or lost during aging.

Publications:

Starke, P.E., Oliver, C.N., and Stadtman, E.R.: Modification of Hepatic Proteins in Rats Exposed to High Oxygen Concentration. The FASEB Journal 1: 36-39, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00263-02 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Regulation of Phosphatidylinositol-specific Phospholipase C

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

| | Name | Title | Affiliation |
|---|---|---|---|
| PI: | Sue Goo Rhee | Research Chemist | LB, NHLBI |
| | Won Chul Choi | Guest Worker | LB, NHLBI |
| | Kee Young Lee | Visiting Fellow | LB, NHLBI |
| | Pann-Ghill Suh | Visiting Fellow | LB, NHLBI |
| | Sung Ho Ryu | Visiting Fellow | LB, NHLBI |
| | Sang Yeol Lee | Visiting Fellow | LB, NHLBI |

COOPERATING UNITS (if any)

Dr. Charles R. Gerfen, Laboratory of Cell Biology, NIMH

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5.6 | 5.3 | 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 hydrolysis of phosphoinositides by phospholipase C is a major pathway of signal transduction during the response of many cells to stimulation by hormones, peptide growth factors, neurotransmitters, and other regulatory ligands. (1) Three distinct phospholipase C isozymes (PLC-I, II, and III) were purified to homogeneity from bovine brain. Their apparent MW determined under a denaturing condition were 150,000, 145,000 and 85,000 for PLC-I, II, and III, respectively. PLC-I and III mainly exist in dimeric form, whereas PLC-II is present predominantly as monomer. (2) The catalytic properties of the 3 isozymes were studied by using small unilamellar vesicles prepared from either phosphatidylinositol (PI) or phosphatidylinositol-4,5-bisphosphate (PI-bisphosphate) as substrate. Hydrolysis of both PI and PI-bisphosphate by the 3 enzymes was dependent on calcium. However, at low calcium concentration, PI-bisphosphate was the preferred substrate for all 3 enzymes. With PI as substrate, the order of specific activity was PLC-III > PLC-I. Contrarily, the order was PLC-I > PLC-III > PLC-II for PI-bisphosphate hydrolysis, meaning that PLC-I is the most specific for PI-bisphosphate. (3) Both polyclonal (rabbit) and murine monoclonal antibodies were prepared against the 3 isozymes. Antibodies derived against one form of the isozymes did not cross-react with another form, indicating that the 3 isozymes are immunologically independent. (4) The subcellular distribution of PLC-I and PLC-II in brain homogenates was measured using radioimmunoassay. More than 90% of PLC-II was found in the cytosolic fractions, whereas the PLC-I was equally distributed between cytosolic and membrane fractions. (5) The distribution of PLC-I and PLC-II was studied in 17 different rat organs and 20 different cultured cells. PLC-I is specific to brain, whereas PLC-II is widely distributed in various tissues and cells. (6) Monoclonal antibodies were used to map the distribution of PLC-I and PLC-II in rat brain using immunohistochemical techniques. Both forms of PLC appear to be distributed widely but in specific neuronal systems. In some systems, both forms of the enzymes appear to be co-localized. However, in other systems, PLC-II appears to be expressed whereas PLC-I is not.

Project Description

Objectives:

Interaction of ligands with $Ca^{2+}$-mobilizing receptors stimulates the phosholipase C (PLC)-mediated hydrolysis of phosphatidyl-inositol-4,5-bisphosphate and generates two second messenger molecules, diacylglycerol and inositol-1,4,5-trisphosphate. Diacylglycerol remains in the plasma membrane and activates protein kinase C, while $IP_3$ causes the release of $Ca^{2+}$ from endoplasmic reticulum.

The object of our studies is to understand how the receptor function is coupled to phospholipase C activity.

Major Findings:

1. Purification of Phospholipase C Isozymes.

It had been shown by a number of workers that phospholipase C activity in mammalian brain can be resolved into several fractions. We, for the first time, purified three phospholipase C (PLC-I, II, and III) isozymes from bovine brain to homogeneity. After several column chromatography steps involving ion exchange chromatography on DE52, heparin-agarose chromatography, reverse phase chromatography on TSK phenyl 5PW and ion exchange chromatography on MonoS column, 2.1 mg of PLC-I, 2.4 mg of PLC-II, and 0.6 mg of PLC-III were obtained from 48 bovine brains. Their apparent molecular weights determined under a denaturing condition were 150,000, 145,000, and 85,000 for PLC-I, II, and III, respectively. PLC-I and PLC-III mainly exist in dimeric form, whereas PLC-II is present predominantly as monomer.

2. Catalytic Properties.

The catalytic properties of the three isozymes were studied by using small unilamellar vesicles prepared from either phosphatidylinositol (PI) or phosphatidylinositol-4,5-bisphosphate ($PIP_2$) as substrates.

pH dependence: All three enzymes exhibited pH optima at acidic pH, around 4.8 for PLC-I, 5.0 for PLC-II, and 5.5 for PLC-III. As the pH increased, PLC-I activity decreased rapidly, and PLC-II less rapidly, while PLC-III activity decreased only by 30% and remained constant between pH 7.0-8.5. The specific activities of the three enzymes were similar at their optimal pH. However, the order of specific activity was PLC-III > PLC-II > PLC-I at neutral pH.

$Ca^{2+}$ dependence: With unilamellar vesicles of PI as substrate, the hydrolysis rates of PLC-II and PLC-III increased with increasing $Ca^{2+}$ concentration up to 10 mM, while the activity of PLC-I increased slowly until it reached a maximum at 6 mM $Ca^{2+}$ and then decreased. Although the PI-hydrolyzing specific activities of the three enzymes were similarly low below 10 μM $Ca^{2+}$, the difference became significant at higher concentrations of $Ca^{2+}$. For example, the specific activities observed with enzymes I, II and III were 2, 8, and 24 μmol/min/mg, respectively, at 10 mM $Ca^{2+}$. With $PIP_2$ vesicles as substrate,

the rates for the three enzymes increased with increasing $Ca^{2+}$ concentration until they reached maxima at 10 ~ 100 μM. $Ca^{2+}$ at concentrations higher than 100 μM inhibited the activities, perhaps owing to precipitation of the substrate. At all $Ca^{2+}$ concentrations, the specific activities were PLC-I > PLC-III > PLC II. At 0.1-1 μM $Ca^{2+}$, the $PIP_2$-hydrolyzing activities of PLC-I and PLC-III were much higher than their PI-hydrolyzing activities. For example, at 1 μM $Ca^{2+}$, the polyphosphoinositide hydrolysis rates were 5.0 and 2.8 μmol/min/mg for PLC-I and PLC-III, respectively, while the PI-hydrolysis rate was 0.3 μmol/min/mg for both enzymes. The rate of $PIP_2$-hydrolysis by PLC-II was 0.35 μmol/min/mg at 0.1 μM $Ca^{2+}$, which is ~ 3-fold of the PI-hydrolysis rate at the same $Ca^{2+}$ concentration; but at 1 μM, the rates for both phosphoinositides were nearly the same.

Effects of heavy metal ions: At a concentration of 50 μM, $Hg^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ inhibited both PLC-I and PLC-II activities by more than 80% while $Cd^{2+}$ inhibited only PLC-II. Other metal ions such as $Co^{2+}$, $Mn^{2+}$, $Mg^{2+}$, and $Ni^{2+}$ were without effect on either PLC-I or PLC-II. Weak inhibitions of PLC-I and PLC-II were observed with 50 μM $Fe^{3+}$. PLC-II was extremely sensitive to $Hg^{2+}$ ion. Fifty percent inhibition occurred at 0.2 μM ($I_{0.5}$ = 0.2 μM). PLC-I was less sensitive to $Hg^{2+}$ ($I_{0.5}$ = 1 μM). $Cd^{2+}$ inhibited PLC-II ($I_{0.5}$ = 5 μM) but not PLC-I. Inhibition by $Zn^{2+}$ and $Cu^{2+}$ was also more pronounced for PLC-II than for PLC-I: $I_{0.5}$ of $Zn^{2+}$ was 4 μM for PLC-II and > 10 μM for PLC-I; $I_{0.5}$ of $Cu^{2+}$ was 2 μM for PLC-II and 10 μM for PLC-I. We further investigated whether the inactivation caused by heavy metals can be reversed either by DTT or EDTA. The activities of PLC-I and PLC-II could be restored completely if after inactivation with $Hg^{2+}$ they were incubated with DTT, while similar treatment with EDTA had no effect. In contrast, the inhibition by $Zn^{2+}$ and $Cd^{2+}$ could be reversed completely by EDTA but not by DTT. The $Cu^{2+}$-mediated inhibition was partially reversed by either DTT or EDTA.

3. Immunological Properties.

Murine monoclonal antibodies directed against PLC-I and PLC-II were prepared. The competition experiments showed that anti-PLC-I antibodies, K-32-3, K-82-3, and K-92-3, recognize different epitopes on PLC-I and that anti-PLC-II antibodies, A-2-5, B-12-5, E-8-4, B-16-5, D-7-3, and E-9-4 bind to different sites on PLC-II. Specificity of these antibodies to either PLC-I or PLC-II could be shown by binding experiments with immunoaffinity gels. Each of the 3 immunoaffinity gels prepared separately from the anti-PLC-I antibodies retained > 95% of PLC-I activity while less than 5% of PLC-II activity was retained by them. On the other hand, the immunoaffinity gels prepared separately from the 6 anti-PLC-II antibodies could retain PLC-II (~ 100%) but not PLC-I.

Purified PLC enzymes exhibited protein bands corresponding to a $M_r$ of 150,000 and 145,000 for PLC-I and PLC-II, respectively, when analyzed on a SDS-polyacrylamide gradient gel. Immunoblots of the SDS-PAGE gel were carried out by using individual monoclonal antibodies as well as their mixtures. Each of the 3 anti-PLC-I antibodies, K-32-3, K-82-3, and K-92-3, independently recognized 150 KDa PLC-I polypeptide but not 145 KDA PLC-II. Similarily, each of the 6 anti-PLC-II antibodies, A-2-5, B-12-5, E-8-4, B-16-5, D-7-3, and E-9-4, was specific to 145 KDa PLC-II polypeptide.

Monoclonal antibodies against PLC-III is not characterized yet, but experiments with anti-PLC-III polyclonal antibody indicate that PLC-III is also distinct from PLC-I and PLC-II.

4. Phospholipase C Associated with Membrane Fractions of Bovine Brain.

The subcellular distribution of PLC-I and PLC-II in brain homogenates was measured using radioimmunoassay. Significant differences were found in the distribution of the two forms of PLC in 100,000 xg supernatants (cytosolic fraction) of brain homogenized in hypotonic buffer and 2 M KCl extracts of washed pellets (particulate fraction). More than 90% of PLC-II was found in the cytosolic fractions, whereas the PLC-I-like molecules were equally distributed between cytosolic and particulate fractions. Purification of PLC enzyme to near homogeneity from the particulate fractions yielded two PLC enzymes both of which could be recognized by anti-PLC-I antibodies but not by anti-PLC-II antibodies. Their apparent molecular weights determined under a denaturing condition were 150,000 and 140,000. The polypeptide of the 150 KDa enzyme seems to be the same as that of the cytosolic enzyme PLC-I: their molecular weights were identical and their trypsin-digested peptides yielded a very similar elution profile on a C18 reverse phase column. It is proposed, therefore, that PLC-I and its truncated form are weakly associated with membranes.

5. Distribution of PLC-I and PLC-II in Various Rat Organs and in Cultured Cells.

Radioimmunoassay was used to quantitate PLC-I and PLC-II in various rat organs and in cultured cells. The results in Tables 1 and 2 suggest that PLC-I is very specially localized to brain while PLC-II is widely distributed.

Table 1. Estimation of PLC I and II in Various Rat Organs by Radioimmunometric Assay

| | quantity of PLC (ng/mg of protein) | |
|---|---|---|
| organs | PLC I | PLC II |
| 1. brain | 794.4 | 200.1 |
| 2. pancreas | 3.7 | 41.1 |
| 3. seminal vesicle | 9.6 | 40.9 |
| 4. submaxillary gland | ND | 5.4 |
| 5. liver | 3.4 | 63.4 |
| 6. kidney | 6.0 | 69.5 |
| 7. spleen | 0.2 | 112.0 |
| 8. lung | 1.3 | 83.3 |
| 9. testis | 2.8 | 78.4 |
| 10. ovary | 0.9 | 144.0 |
| 11. uterus | 4.1 | 64.2 |
| 12. bladder | 1.9 | 46.2 |
| 13. stomach | 2.2 | 65.4 |
| 14. heart | 1.5 | 51.4 |
| 15. diaphram | 0.2 | 14.6 |
| 16. leg muscle | ND | 7.9 |
| 17. human platelets | ND | 2.6 |

Table 2. Quantitation of PLC Enzymes in Various Cells by Radioimmunometric Assay

| | quantity of PLC (ng/mg of protein) | | |
|---|---|---|---|
| cell | PLC-I | PLC-II | Remarks |
| NIE 115 | ND | 239.5 | neuroblastoma |
| NS 20Y | ND | 129.1 | " |
| N18 TG-2 | ND | 138.2 | " |
| $N_4$(K-142) | 1.4 | 221.7 | " |
| $N_8$(K-130) | ND | 183.4 | " |
| $C_6$Bu-1 | 72.1 | 259.4 | rat glioma |
| $C_6$ATCC | 119.0 | 205.3 | " |
| 1321N1 | 40.6 | 579.8 | astrocytoma |
| NG108-15 | 1.6 | 189.2 | N18 TG-2 X $C_6$Bu-1 |
| NH108-15 | 1.7 | 213.7 | theophylline + $PGE_1$ |
| NBr 10A | ND | 132.0 | N18TG-2 X BRA-30E |
| NCB 20 | ND | 149.9 | N18TG-2 X CHEB |
| PC12-C | ND | 240.1 | pheochromocytoma |
| RBL-3H2 | ND | 65.5 | basophilic leukemia |
| NIH 3T3 | ND | 462.0 | fibroblast |
| NCI-H82 | ND | 256.3 | lung cancer |
| NCI-H209 | ND | 341.6 | lung cancer |
| $SP_2$ | ND | 95.0 | mouse myeloma |
| P815($H-2^d$) | ND | 190.3 | mastcytoma |
| BW5147($H-2^k$) | ND | 338.1 | thymoma |
| rat brain | 794.4 | 200.1 | whole brain |

BRA-30E: buffalo rat liver cell.
CHEB: Chinese hamster embryo brain cell.

6. Immunohistochemical Localization of PLC-I and PLC-II Isozymes in Neuronal Systems in the Brain.

Monoclonal antibodies which distinguish between PLC-I and II were used to map their distribution in the rat brain using immunohistochemical techniques. Both forms of PLC appear to be distributed widely but in specific neuronal systems. In some systems, both forms of the enzyme appear to be co-localized, however, in other systems, PLC-II appears to be expressed, whereas PLC-I is not. The immunoreactive distribution of both PLC-I and PLC-II is similar in the cortex, being present in neurons in most neocortical areas and in the hippocampal pyramidal and dentate gyrus granule cells. The densest labeling of both PLC isozymes is in the basal ganglia, in the medium spiny projection neurons of the striatum. Although both PLC isozymes labeled these neurons, labeling with monoclonal antibodies to PLC-I produced denser staining. Both PLC-I and PLC-II immunoreactivity was evident in the striatopallidal and straitonigral projections and terminals. However, PLC-II but not PLC-I immunoreactivity was also present in the neuronal targets of these projection systems, in both the globus pallidus and the substantia nigra. In the thalamus, both PLC-I and II immunoreactivity is expressed. In some nuclei both forms are found; in the thalamic reticular nucleus, the mediodorsal thalamic nucleus, whereas PLC-I appears to be restricted to these thalamic nuclei, PLC-II is more broadly expressed in the thalamus. In particular, neurons in the medial habenula express PLC-II but not

PLC-I, consistent with the labeling by PLC-II in the teminal fields in the interpeduncluar necleus. Within the hypothalamus PLC-I and II also have different distributions. PLC-I immunorectivity is absent in the paraventricular nucleus, whereas PLC-II immunoreactivity is present in a subpopulation of neurons, most likely a subset of parvocellular neurons.

Proposed Course:

1. Identification of guanine nucleotide binding protein(s) which couple receptor function to phospholipase C. Recent studies by a number of investigators suggest that the coupling of receptor function to PLC is mediated by unspecified G protein(s). Using the purified three PLC isozymes, reconstitution experiments will be pursued for the G protein(s).

2. Studies on the distribution of PLC-III in various rat organs, in o ultured cells and in neuronal systems. We recently obtained monoclonal antibodies against PLC-III. Using these antibodies, immunoassays will be developed to quantitate or map PLC-III in various tissues and cells, as we did with PLC-I and PLC-II.

3. Nucleotide sequence of cDNA encoding PLC-I, II, and III isozymes. $\lambda gt_{11}$ library was screened for those CDNA. Clones for PLC-I and II were isolated and sequence is in progress.

Publications:

Rhee, S.G., Bang, W.G., Kim, K.H., Koo, J.H., Min, K.H., Park, S.C., and Son, H.S.: Regulation of glutamine synthetase in E. coli and S. cerevisiae. In Proceedings of Science, Korea, 1986, pp. 112-117.

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Son, H.S. and Rhee, S.G.: Cascade Control of Escherichia coli glutamine synthetase: Purification and properties of $P_{II}$ protein and nucleotide sequence of its structural gene. J. Biol. Chem. 262: 8690-8695, 1987.

Rhee, S.G., Bang, W.G., Park, S.C., Koo, J.H., and Min, K.H.: Regulation of glutamine synthetase activity and the repression of its biosynthesis in E. coli are mediated by two nucleotidylation/denucleotidylation cycles. In Chock, P.B., Tsou, L., and Huang, C.Y. (Eds.): Dynamics of Soluble and Immobilized Enzyme. Amsterdam, Springer-Verlag, 1987, in press.

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Lee, K.-Y., Ryu, S.H., Suh, P.-G., Choi, W.C., and Rhee, S.G.: Phospholipase C associated with particulate fractions of bovine brain. Proc. Natl. Acad. Sci. U.S.A., in press, 1987.

Ryu, S.H., Suh, P.-G., Cho, K.S., Lee, K.-Y., and Rhee, S.G.: Bovine brain cytosol contains three forms of immunologically distinct phosphoinositide-specific phospholipase C. Proc. Natl. Acad. Sci. U.S.A., in press, 1987.

Kim, K.H. and Rhee, S.G.: Subunit interaction elicited by partial inactivation with L-methionine sulfoximine and ATP differently affects the biosynthetic and γ-glutamyltransferase reactions catalyzed by yeast glutamine synthetase. J. Biol. Chem., in press, 1987.

Ryu, S.H., Lee, S.Y., Lee, K.-Y., and Rhee, S.G.: Catalytic properties of inositol trisphosphate kinase: Activation by $Ca^{2+}$ and calmodulin. FASEB J. in press, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00265-01 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Factors Affecting Expression of a Selenium-Containing Enzyme

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

| | | | |
|---|---|---|---|
| PI: | Milton J. Axley | Staff Fellow | LB, NHLBI |
| Others: | Thressa C. Stadtman | Section Chief | LB, NHLBI |

COOPERATING UNITS (if any)

Dr. August Böck, University of München, München, West Germany.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.4 | 1.1 | 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.)

The metal selenium is required at low levels in the diets of all mammals, including humans. Recent evidence has indicated selenium may play a role in cancer prevention. Mammals, birds, and several species of bacteria incorporate selenium as selenocysteine at specific sites of a few proteins. In all known cases, the messenger RNA codon for selenocysteine is UGA, which normally is a stop codon terminating synthesis of the polypeptide chain. The mechanism by which selenocysteine is incorporated into protein remains a mystery. The bacterium Escherichia coli produces a selenocysteine-containing enzyme, formate dehydrogenase, under anaerobic conditions in the absence of nitrate. We have used this as an easily manipulable model system for analyzing the components necessary for selenocysteine incorporation. The gene for formate dehydrogenase has been cloned and isolated by others. Fusions of the DNA sequences controlling formate dehydrogenase expression with the gene for an easily detectable enzyme marker, β-galactosidase, have provided a simple assay system for factors affecting formate dehydrogenase synthesis. We have found that in vivo expression of formate dehydrogenase is increased under conditions where the DNA is less supercoiled. This is in direct contrast to the results found for other anaerobically induced genes. An in vitro system for translation of the formate dehydrogenase gene is being constructed. This will allow identification of the biochemical machinery for selenocysteine incorporation.



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

Project Description:

Objectives: Selenium is an essential nutrient for mammalian species. Mammals, birds, and some bacteria synthesize proteins which contain selenocysteine residues as specifically required components of the polypeptide backbone. The objective of this study is to discern the biochemical mechanisms which govern selenium incorporation into polypeptide.

Major Findings:

The gene for the selenium-containing protein formate dehydrogenase of *Escherichia coli* has been cloned in the laboratory of August Böck (University of München). Fusions of the gene to the β-galactosidase structural gene have provided a facile assay for factors affecting formate dehydrogenase expression. Recent reports have suggested that bacterial genes expressed under anaerobic conditions require the activity of the enzyme DNA gyrase, which increases the supercoiling of DNA. In contrast to these reports, we found that inhibition of gyrase activity enhances the expression of formate dehydrogenase, an anaerobically expressed gene. This was found by observing formate dehyrogenase:β-galactosidase fusion gene synthesis when gyrase activity was inhibited with any of three chemical inhibitors or a temperature-sensitive mutant.

An *in vitro* system for synthesis of formate dehydrogenase would allow dissection of the essential biochemical components for selenium utilization. Such a system is presently being developed in our laboratory. The fusion gene has been synthesized *in vitro* using a cell-free extract from wild-type *E. coli* cells.

Significance to Biomedical Research and the Program of the Institute:

Selenium is a required mineral in the human diet, and it is thought to have a role in cancer prevention. Elucidation of the biochemical mechanism of selenium utilization would allow a greater appreciation of the proper role for selenium in the diet.

Additionally, the amino acid selenocysteine could play an important role in protein engineering. Cysteine residues are involved in the catalytic action of many enzymes. Replacement of cysteine by selenocysteine could significantly alter the catalytic properties of an enzyme without disrupting the structure of the protein. With an understanding of the mechanism of selenocysteine incorporation into protein, one could direct the mutagenesis of a protein such that selenocysteine replaces cysteine.

Proposed Course:

Extracts from an *E. coli* mutant unable to incorporate selenium into polypeptide will be used for construction of an *in vitro* transcription-translation system. Such a system will be unable to synthesize fusion genes containing the selenocysteine codon. Addition of extracts of wild-

type cells will restore the ability to incorporate selenium and thus synthesis of the fusion protein. This provides an assay for isolation and identification of biochemical components essential for selenium incorporation.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00266-01 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Cloning of Selenoprotein A Gene From Clostridium sticklandii

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

| | | | |
|---|---|---|---|
| PI: | Gregory E. Garcia | Staff Fellow | LB, NHLBI |
| Others: | Thressa C. Stadtman | Section Chief | LB, NHLBI |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.4 | 1.1 | 0.3 |

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☐ (a1) Minors

☐ (a2) Interviews

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


Selenoprotein A of the glycine reductase complex from Clostridium sticklandii has been purified and partially sequenced around the selenocysteine residue. Two oligonucleotide probes to this region have been synthesized and used to screen a C. sticklandii gene library to select for the selenoprotein A gene.

The probes have proven to be relatively non-specific for colony selection of the gene. Each probe has selected for a different set of clones from the library. The selection has required conditions of low stringency. From 30 clones selected by one of the probes, three have been sequenced and determined not to code for the selenoprotein A gene.

Southern blot analysis of clostridial DNA digested to completion with the restriction enzyme Hind III has shown that the probes hybridize primarily to single but different DNA fragments. This suggests a strategy of isolating the fragments for cloning to provide an enriched library for selection of the selenoprotein A gene.

Project Description:

Objectives: Selenium is specifically incorporated as selenocysteine into a number of proteins found in humans, other higher animals, and several bacterial species. The mechanism of incorporation is unknown. Selenoprotein A of *Clostridium sticklandii* has been purified and the sequence around the essential selenocysteine has been determined to be: CysPheVal-SecThrAlaAlaGlyMetAspLeuGluAsnGluLys. Oligonucleotide probes complimentary to the deduced mRNA sequence have been synthesized and used to screen a *C. sticklandii* gene library in order to isolate the gene for selenoprotein A. Isolation of the selenoprotein A gene will enable the determination of the remaining amino acid sequence of selenoprotein A and provide the basis for the investigation of the specific incorporation of selenium into protein.

Major Findings:

Selenoprotein A isolated from *C. sticklandii* has been shown to contain selenium in the form of selenocysteine. The partial amino acid sequence around the selenocysteine has been determined to be: --CysPheValSecThrAlaAla-GlyAlaMetAspLeuGluAsnGluLys--. Two overlapping oligonucleotide probes complimentary to the deduced mRNA sequence of the peptide were synthesized. The first probe is a 33mer oligonucleotide and contained the sequence from the Thr to the C-terminal Lys. The second probe contained 20 nucleotides complimentary for the Met to the C-terminal Lys. Where the redundancy of the genetic code predicted any of the four nucleotides at a position, an inosine was incorporated into the probe sequence. Each probe has a degeneracy of 16. The two probes were used to screen a *C. sticklandii* gene library.

Genomic DNA was isolated from *C. sticklandii*. The DNA was partially digested with restriction enzyme Sau 3AI. The DNA was fractionated by linear density sucrose gradient ultracentrifugation. DNA fractions of 6,000 to 2,000 bps were collected and homopolymer tailed with deoxycytidines. The tailed DNA was annealed with deoxyguanidine tailed pBR322 digested Pst I and cloned into *Escherichia coli* strain RR1. The library consisted of 10,000 clones and contained sufficient clostridial DNA for eight copies of the selenoprotein A gene.

Each probe selected a different set of 30 to 40 clones from the library. The hyridization conditions required were of low stringency. The hybridization temperatures in 0.3 molar sodium citrate and 0.03 molar sodium chloride (2XSSC) were 15 to 20 degrees Celsius below the calculated Td for 100% complimentary. Three clones selected by the 20mer oligonucleotide probe were sequenced and determined to be different from each other and not to code for the selenoprotein A gene. These results indicate that the internal inosine bases destabilize the hybridization of the remaining probe sequence. The destabilization did not allow selection of the selenoprotein A gene from the clostridial DNA library prepared as described. This prompted the investigation of creating a library of clostridial DNA that would be enriched for sequences that hybridize to the probes.

Southern blots of genomic clostridial DNA, completely digested by restriction enzyme Hind III, were analyzed by hybridization with the probes. The two probes hybridize to different restriction fragments. The 33mer oligonucleotide probe hybridizes to a fragment of 5,000 bps and weakly to a number of shorter fragments. The 20mer oligonucleotide probe hybridizes to a fragment of 3,000 bps and weakly to three other fragments. The hybridization temperatures in 2XSSC for the Southern blots are slightly higher than the temperature for hybridization used for colony selection from the original gene library.

Proposed Course:

A new gene library of clostridial DNA that is enriched for sequences that hybridize to the 20mer oligonucleotide probe will be created. This library will be used to isolate the gene for selenoprotein A.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00267-01 LB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Isolation of Isopeptidase, An Enzyme Regulating Selective Protein Degradation

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

| | | | |
|---|---|---|---|
| PI: | James C. Cook | Staff Fellow | LB, NHLBI |
| Others: | P. Boon Chock | Section Chief | LB, NHLBI |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.85 | 0.75 | 0.1 |

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☐ (a1) Minors

☐ (a2) Interviews

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

I have begun the groundwork for purification of an enzyme, isopeptidase, which is a component of the ubiquitin-ATP-dependent proteolytic pathway in eukaryotes. The role of this enzyme is not fully understood, but it appears to act antagonistically with the degradative process by removing ubiquitin from protein-ubiquitin conjugates thus preventing the degradative step of this pathway. The level of isopeptidase could serve as a regulator of the level of ubiquitinated proteins and thus the level of protein degradation, and it could serve a proof-reading function by removing ubiquitin from protein not destined for degradation.

Work-to-date has been directed toward construction of an assay for isopeptidase. Synthesis of a known substrate, ubiquitin-lysozyme, has been successful on a small scale, but precipitation of the product has plagued attempts to scale up the reaction.

Initial attempts to purify the synthetic conjugate from unreacted protein indicate that CM-cellulose chromatography should be useful.



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

Project Description:

Objectives: To purify isopeptidase for biochemical characterization. This knowledge should help define the role of this enzyme in regulating the level of ubiquitin-ATP-dependent protein degradation, and it may also reveal the role of a new cyclic cascade involving ubiquitination/deubiquitination of proteins.

Major Findings:

In order to purify isopeptidase, a suitable assay must be constructed. Requirements of the assay include synthesis of a substrate and a means of detecting the progress of the reaction.

A combined synthetic/biosynthetic method has been chosen for the synthesis of a ubiquitin-lysozyme conjugate to serve as a substrate for the isopeptidase reaction. Both ubiquitin and lysozyme are available commercially and a published protocol holds promise for yielding milligram quantities of substrate.

Subsequent detection of substrate and reaction products could be accomplished by fluorescence labeling of one or both of the conjugated moieties.

I. Fluorescent Labeling of Substrate.

The fluorescent labeling of lysozyme was accomplished by the use of azidofluorescein. This label was chosen because of the non-specific nature of the labeling site. Most available fluorescent probes attach via primary amines which would block the conjugation of lysozyme to ubiquitin in the next step. Success of labeling was determined by co-migration of protein and label on gel filtration columns.

II. Conjugation of Lysozyme to Ubiquitin.

The procedure involves three reaction steps with some purification between them. The first is a trypsin catalyzed transpeptidation reaction using a large molar excess of gly-gly-OEt to selectively modify the c-terminus of ubiquitin to an ethyl ester. Purification of the product is by gel filtration and ion-exchange chromatography. The ubiquitin ethyl ester is treated with hydrazine, dialyzed, and converted to ubiquitin azide by treatment with nitrous acid. Addition of lysozyme in triethylamine allows displacement of the azide by the primary amines of lysozyme forming the ubiquitin-lysozyme conjugate. Although a 30% yield was reported in the literature, several attempts to duplicate the procedure gave a considerably lower yield. This was due, in part, to precipitation of the conjugate after the final reaction step. Precipitation was avoided by modification of the desalting procedure. However, the problem recurred when the reaction was scaled up. Attempts to renature the product have met with limited success probably due to the destabilizing effect of ubiquitin attached to the lysozyme.

III. Purification of Ubiquitin-Lysozyme Conjugate.

Although precipitation of the conjugate was a problem during the scale-up of the reaction, enough of the product survived to allow some initial trial purification procedures. Ion-exchange chromatography with CM-cellulose appears to be a simple and effective way to separate ubiquitin-lysozyme conjugate from unreacted lysosyzme, ubiquitin, and ubiquitin-ubiquitin conjugates.

## Significance to Biomedical Research and the Program of the Institute:

Selective protein degradation by the ubiquitin-ATP-dependent is an important component in the regulation of metabolic activity and has been shown to be a vital process in the maturation of red blood cells. Isopeptidase could play a key role in regulating the level of protein degradation by catalyzing the removal of ubiquitin from ubiquitin-protein conjugates. Characterization of this enzyme and the reaction it catalyzes could shed light on the nature of disease processes in which abnormal metabolism of ubiquitinated protein has been implicated (Alzheimer's Disease).

## Proposed Course:

Conditions are being sought which will provide a better yield from the conjugation reaction. Once this has been accomplished, conjugation of fluorescently labeled proteins will be tried.

Once labeled substrate is available, an assay which will allow rapid screening of samples for isopeptidase activity will be constructed. The assay should be suitable for testing large numbers of column fractions. One possible assay is outlined below. Fluorescently labeled lysozyme is conjugatd to ubiquitin and purified. Due to the large difference in pI of lysozyme and ubiquitin, a proper choice of pH and ionic strength should allow ubiquitin and ubiquitin-lysozyme* to wash through a small CM-cellulose column while unconjugated lysozyme* absorbs. Fluorescence intensity measurement of the non-adsorbed fraction would indicate isopeptidase activity by a decrease in fluorescence relative to a control.

## Publications:

None.

| DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE<br>**NOTICE OF INTRAMURAL RESEARCH PROJECT** | PROJECT NUMBER<br>Z01 HL 00268-01 LB |
|---|---|

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Oxidation of Polymers of Amino Acids and Proteins

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

PI: J. Michael Poston Research Chemist LB, NHLBI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.3 | 1.0 | 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.)*


Polymers of L-proline, L-histidine, and L-lysine have been oxidized using Fenton's reagent with or without added ascorbic acid. Carbonyl groups are introduced into the polymers as a result of the oxidation and efforts are underway to identify these products. Methods used for this purpose include derivatization with p-aminobenzoic acid in the presence of cyanoborohydride, followed by HPLC analysis of the products. In addition, amino acid analysis using phenylisothiocyanate derivatization and HPLC separation has been used. Oxidation of the polymers leads also to the shortening of the polymeric chains by what appears to be random scission.





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

Project No. Z01 HL 00268-01 LB

## Project Description

### Objectives:

Earlier studies in this laboratory have shown that oxygen radical-mediated inactivation of enzymes is associated with the conversion of some amino acid residues to carbonyl derivatives. The purpose of this investigation is to examine the products of oxidation of proteins using polymers of amino acids (homopolymers) as model compounds.

### Major Findings:

Previous studies in this laboratory have shown that proline, arginine, lysine, and histidine residues of proteins are particularly susceptible to oxidation, one of the products of oxidation of proline being 5-oxo-2-amino-pentanoic acid. It was found that the polymer would react with 2,4-dinitro-phenylhydrazine when the poly-L-proline was oxidized using the Fenton's reagent (ferrous iron plus $H_2O_2$). In addition to this production of carbonyl groups, the polymers were found to be less readily precipitable with trichloroacetic acid. When the oxidized polymers were examined using gel filtraiton, they were found to be appreciably shortened, indicating that they had undergone random scission. Oxidation of poly-L-lysine and poly-L-histidine also show decreases in detectable polymer, suggesting scission. These latter polymers have not yet been subjected to gel filtration.

Amino acid analyses were conducted using derivitization by phenylisothio-cyanate (PITC) and subsequent HPLC chromatography. Evidence was obtained upon hydrolysis of oxidized polyproline showing that both *cis*- and *trans*-4-hydroxy-proline were produced. When monomers of *cis*-hydroxyproline were oxidized with Fenton's reagent, *trans*-hydroxyproline, dehydroproline, and proline were formed. Similarly, when the *trans*-isomer was oxidized, *cis*-hydroxyproline, dehydropro-line gave rise to each of the other compounds. Careful examination of the reaction mixtures showed that, while the proportions of these products vary from experiment to experiment, the findings are generally consistent qualitatively.

It was hoped that the carbonyl-containing amino acids would be identifi-able as the PITC derivative. This has not yet proven to be possible. Alter-native methods in which the carbonyl group would be converted to the Schiff's base with an appropriate amine and then reduced with $NaBH_4$ were examined. Dimethylaminobenzylamine was tried because it would have a strong ultraviolet absorption spectrum, but neither $NaBH_4$ nor $NaCNBH_3$ gave rise to a detectable product. On the other hand, p-aminobenzoic acid proved to be an excellent reagent and reduction with $NaCNBH_3$ yields products with good UV absorption. These products are currently being examined.

An interesting discovery during the course of these investigations has been the extraordinary diversity of products of oxidation that result when the chelator ethylenediaminetetraacetic acid (EDTA) is exposed to Fenton's reagent. These are so numerous that minor oxidation products of amino acids can be overlooked or misidentified in their presence. Purification of polymers by repeated precipitation with trichloroacetic acid obviates the problem.

Proposed Course:

Efforts will be continued to identify the products containing carbonyl groups resulting from oxidation of poly-L-proline. In addition, the polymers of L-lysine and L-histidine will be oxidized and examined both for changes in amino acid composition and for Schiff's base formation with p-aminobenzoic.

## ANNUAL REPORT OF THE CARDIOLOGY BRANCH

National Heart, Lung, and Blood Institute

October 1, 1986 through September 30, 1987

The experimental interests of the Cardiology Branch focus on 1) elucidating the mechanisms responsible for dynamic alterations in coronary vascular resistance; 2) examining the possible role aberrations of cytosolic calcium levels play in the development of microvascular angina, hypertrophic cardiomyopathy (HCM) and hypertension; 3) studying the role of angiogenesis in the pathophysiology and treatment of ischemic heart disease; 4) developing intravascular remodeling techniques for the treatment of coronary obstructive disease.

### 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. The recognition of vasospastic angina (Prinzmetal, or variant angina) focused attention on the fact that the large epicardial coronary arteries can spontaneously constrict and that this constriction can be severe enough to precipitate myocardial ischemia. However, the clinical syndrome of variant angina is uncommon. Over the past three years we have explored the possibility that dynamic coronary vasoconstriction may more commonly involve the small intramural coronary arteries.

<u>Microvascular coronary vasoconstriction and angina:</u> 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. The chest pain was also associated with diminished myocardial lactate consumption and abnormalities in LV function. We also demonstrated that the reduced vasodilator reserve occurred not only in response to metabolic stimuli (pacing-induced increase in myocardial $VO_2$), but also to a diminished absolute capacity of the coronary vessels to dilate in response to the potent coronary arteriolar vasodilator, dipyridamole. Further analyses of these data suggested that the flow limitation is due to narrowing of the small pre-arteriolar coronary arteries, rather than of the arterioles, per se. On the basis of these findings, we now refer to this syndrome as microvascular angina (MVA).

<u>Evidence of a diffuse disorder of smooth muscle:</u> Several other intriguing findings evolved from these original studies. Abnormal esophageal motility was found in over half of the pts with MVA, suggesting this syndrome is characterized by a general increase in smooth muscle tone. This concept was further substantiated by examining the peak reactive hyperemic response of the forearm to ischemia. Peak flow was reduced and vascular resistance was higher after 5 minutes of forearm ischemia in pts with MVA compared to an age and sex matched control group. These findings generated two important and novel hypotheses. First, they suggested that pts with MVA have a generalized defect involving an increase in smooth muscle tone. Second, they raised the possibility that a common etiologic link, related to aberrations in cytosolic calcium levels, might exist for three diseases -- the syndrome of MVA, HCM, and hypertension.

ABERRATIONS OF CYTOSOLIC CALCIUM LEVELS AS A CAUSE OF MVA, HCM AND HYPERTENSION

MVA: We previously demonstrated that the voltage-dependent calcium channel blockers, verapamil, nifedipine and diltiazem, improved symptoms in most pts with MVA. Some pts, however, were unresponsive to these agents. We have now shown that lidoflazine, a calcium antagonist that blocks calcium entry at sites other than the voltage-sensitive channel, significantly improves effort tolerance and onset of chest pain in MVA pts who had remained symptomatic despite verapamil, nifedipine, or diltiazem. The drug also increases the low coronary flow response both to pacing stress after ergonovine and to dipyridamole. These results suggest that maximal coronary flow is limited in pts with MVA by vasoconstrictor influences mediated by elevated cytosolic calcium levels, and that the basic abnormality and resulting symptoms are improved by calcium entry blocking drugs.

HCM: We previously reported that over 80% of pts dying with HCM have anatomically abnormal intramural coronary arteries characterized by thickened intima and/or media, often accompanied by luminal narrowing. These abnormal vessels were identified frequently in areas of replacement fibrosis, suggesting a role of small vessel disease, with resulting ischemic injury, in the pathophysiology of HCM. We also found that ergonovine reduced the peak coronary flow response to pacing, and increased coronary resistance. Since no large vessel vasospasm occurred, these findings, similar to those demonstrated in pts with MVA, are compatible with the concept that the small intramural coronary arteries are abnormally responsive to vasoconstrictor influences.

To characterize the ischemia presumed to be present in pts with HCM, we employed thallium-201 single photon emission computed tomography to evaluate myocardial perfusion. We demonstrated that reversible regional perfusion defects, suggestive of ischemia, frequently occur in HCM pts. We also found that over half of the totally asymptomatic pts with HCM we studied had reversible perfusion defects, a prevalence similar to that observed in symptomatic pts. These data suggest that asymptomatic HCM pts experience silent myocardial ischemia. Of note, the large majority of these reversible perfusion defects improved or normalized with verapamil therapy. This observation is compatible with the concept that abnormal calcium fluxes cause constriction of the abnormal small coronary arteries present in HCM, leading to ischemia, and thereby to angina and to myocardial necrosis and fibrosis.

Since verapamil exerts its physiologic effects by blocking the voltage sensitive calcium channel, we tested the hypothesis of whether an abnormal density of voltage sensitive calcium channels exist in the hearts of pts with HCM. To explore this possibility we measured the density of dihydropyridine binding sites present in the hearts of HCM pts undergoing surgery, and compared these results with those obtained in non-HCM pts. We found that the density of dihydropyridine binding sites, and presumably therefore of voltage sensitive calcium channels, was elevated in HCM pts. The density of beta adrenergic receptors was the same in both groups. These findings strongly suggest that a specific increase in voltage sensitive calcium channels plays an important primary role in the pathophysiology of HCM.

The functional abnormalities of the small coronary arteries of HCM pts resemble those of MVA pts. To determine whether HCM pts also have a more generalized disorder of vascular smooth muscle vasodilator reserve, we employed plethysmography to study the hyperemic response to forearm ischemia. As with the MVA pts, HCM pts had lower flow responses following ischemia, and higher minimal vascular resistance. These findings therefore suggest that, as in MVA pts, HCM pts also have a generalized disorder of smooth muscle tone that decreases the vasodilator reserve of both the peripheral and coronary arteries and that this defect appears to be caused, at least in part, by increased cytosolic calcium levels.

Hypertension: Since peripheral vasoconstriction is the cause of the elevated blood pressure of hypertensive pts, and since many hypertensive pts experience angina in the absence of large vessel coronary disease, we examined the hypothesis that hypertensive pts also have an abnormality of the coronary microcirculation similar to that observed in MVA and HCM pts. Our studies demonstrated that many pts with hypertension, angina, and normal coronary angiograms exhibit a dynamic abnormality of coronary vasoconstrictor tone at the microvascular level that appears to account for their angina. We also demonstrated that LV diastolic function is abnormal in such pts, and that this abnormality improves in response to the calcium antagonist verapamil, but not to nitroprusside. These results suggest a role for abnormal cytosolic calcium levels in producing the cardiac abnormalities present in hypertension.

As a result of these studies, we hypothesize that aberrations in the control of calcium fluxes, leading to elevated cytosolic calcium levels, can cause diseases heretofore considered etiologically distinct. If the elevated cytosolic calcium levels predominate in the systemic arterioles, hypertension results. If it predominates in the myocardium, the syndrome we now refer to as HCM develops. If it predominates in the coronary microvasculature, we recognize the syndrome of MVA. And if it predominates in the smooth muscle of the esophagus, pts experience esophageal motility disorders. The facts that 1) many pts with MVA have esophageal motility disorders and have abnormal peripheral vascular responses to stress, 2) many HCM pts have hypertension, and 3) many hypertensive pts have MVA, further suggest that these diseases are not etiologically distinct, but share common pathophysiologic mechanisms, which we hypothesize ultimately result in abnormally elevated cytosolic calcium levels. Which tissue, or tissues, develop elevated cytosolic calcium levels will determine the precise clinical presentation.

## HYPERTROPHIC CARDIOMYPATHY

Progression of hypertrophy in HCM: Hypertrophy is one of the primary abnormalities in HCM. To determine whether this occurs throughout the course of the disease and is an important determinant in changing symptoms, we previously performed serial studies in HCM pts at various ages. We found that progressive hypertrophy occurred frequently in children with HCM, being particularly marked during their adolescent growth spurt. Several pts were found to develop hypertrophy de nova during this period, having had normal echocardiographic studies prior to the age of 10 years. To further characterize the genesis of hypertrophy in these children, we analyzed the ECGs of these pts. All 7 who developed de nova hypertrophy during their adolescent growth spurt had abnormal ECGs prior to the appearance of LVH on 2-D echo. Hence, a primary abnormality in HCM exists prior to the appearance of LVH.

We also found that, despite the high frequency of progression of hypertrophy in children, no progression in LV wall thickness was observed in HCM pts over age 23 years. Instead, approximately 15% of adult HCM pts developed a substantial decrease in wall thickness, usually associated with mild LV dilatation, LV wall thinning, decreased ejection fraction, and severe symptoms. Attempts to elucidate the cause of these changes may provide insight as to the basic etiologic mechanisms present in HCM.

Electrophysiologic abnormalities in HCM: Although ventricular tachycardia (VT) occurs on ambulatory monitoring in about 20% of HCM pts, and over 25% of these pts die over a 3 year period, further identification of high risk subgroups has not been achieved. To determine the role of programmed electrical stimulation (PES) in both defining factors contributing to major clinical events and directing therapy, we studied 50 HCM pts with cardiac arrest, syncope, near-syncope, or asymptomatic nonsustained VT. Although over 30% of the pts had inducible VT in the PES laboratory, no pt with asymptomatic nonsustained VT was inducible. A probable electrophysiologic abnormality contributing to clinical symptoms was identified in over 50% of the remaining pts. These data indicate that 1) asymptomatic nonsustained VT may represent lower risk subgroups, and 2) PES frequently identifies potential mechanisms of syncope or cardiac arrest, and therefore may be useful in directing therapeutic strategies.

Amiodarone as a potential cause of sudden death in HCM: We previously demonstrated that amiodarone often improves symptoms markedly in severely limited HCM pts. However, the follow-up data of these pts suggest that this drug may predispose to sudden death. We have treated 72 HCM pts with amiodarone and followed as part of two study protocols: Group 1 (n=50) pts with refractory cardiac symptoms, Group 2 (n=22) pts believed to be at high risk of sudden death because of arrhythmias. Baseline characteristics for the two groups were similar except for functional class. (1= 3.3, vs. 2=2.0). Despite improved symptoms in Group 1, and reduced frequency of nonsustained VT on Holter in both groups, 8 pts died suddenly during the follow-up period. Importantly, sudden death occurred less than 6 months after initiating amiodarone in all Group 1 pts (6/50), and in 2 pts was associated with new nonsustained VT demonstrated on Holter. In Group 1 pts, an early fall in peak filling rate during amiodarone predicted subsequent sudden death. In Group 2 pts, sudden death occurred later (greater than 6 months), was less frequent (2/22) and was unrelated to changes in Holter or LV diastolic function. We conclude that in severely symptomatic HCM pts, amiodarone does not prevent, and may increase the frequency of sudden death, presumably by a proarrhythmic action that is possibly potentiated by a deterioration in LV diastolic function.

NEW APPROACHES TO THE TREATMENT OF ANGINA PECTORIS

Approximately 2-3 years ago we recognized that one of the most important current problems in cardiology is how to improve blood flow to the heart of pts whose own coronary arteries are extensively diseased by severe atherosclerosis, and who have chronic refractory ischemic symptoms no longer responsive to pharmacologic therapy, or amenable to coronary bypass surgery. We therefore initiated studies to develop alternative approaches of treating these pts.

Myocardial Neovascularization: Oncologic research has determined that increases in certain solid tumor cell populations must be preceded by an increase in new capillaries that converge upon the tumor and supply it with blood. This hypothesis implies that angiogenesis is a rate limiting step to most solid neoplasms. It also led to studies seeking to identify those factors responsible for neovascularization (and therefore tumor expansion) with the ultimate hope of developing substances that would inhibit angiogenesis (and thus tumor growth). We were intrigued by the thought that we might employ an analogous but opposite approach: to use angiogenic factors to promote rather than inhibit blood vessel growth in ischemic myocardium. We have therefore initiated studies to determine whether it would be possible to potentiate angiogenesis in ischemic myocardium. Our studies involve two parallel lines of investigation.

Heparin affinity chromatography has allowed the purification from brain and tumors of a family of polypeptides of MW 15-18,000 that are angiogenic in vivo, chemotactic for capillary endothelial cells in vitro, and mitogenic for capillary endothelial cells, fibroblasts and smooth muscle cells in vitro. To determine whether such factors are present in the ischemic heart, we excised the infarct zones of foxhounds 3 hours after LAD coronary artery ligation. After suitable preparation, the processed pellets were run twice through a heparin-sepharose column and eluted with NaCl gradients. Fractions eluted from 1-2M NaCl produced cell growth and increased incorporation of thymidine into 3T3 cells. Electrophoresis showed seven bands, from 14-18,000. These fractions immuno-reacted by slot-blot with a polyclonal antiserum against the end terminus of bFGF. A third heparin column elution yielded a single band at 15,000, which was mitogenic but not immunoreactive. Thus, infarcted myocardium yields several mitogenic polypeptides, one of which is similar if not identical to bFGF.

In another model of myocardial ischemia progressing to infarction, we removed the hearts of 8 rats and incubated them at 24°C for 60 min. Radio-immunoassay, using a polyclonal antiserum against bFGF revealed that ischemia increased bFGF levels. Mitogenicity of the tissue extracts on 3T3 fibroblasts was also significantly higher than in controls. We therefore conclude that ischemia increases bioavailability of bFGF, which then may play a role in coronary angiogenesis or in the healing of infarction. Although bFGF could be synthesized and released by ischemic myocardial cells, we hypothesized that pre-existing stores of bFGF are released from the extracellular matrix and made more bioavailable by myocardial acidosis. To test this concept we extracted control heart and hearts removed from the body of rats for 60 min (ischemic injured) in pH extraction buffers ranging from 7.5 to 4.5, to determine whether a decrease in pH releases bFGF, and if so what pH simulates the ischemia mediated bFGF release. With decrease in pH, bFGF levels in control hearts rose significantly, toward the levels of the ischemia injured hearts. Below, pH 6.5, bFGF levels in the two groups paralleled each other. We conclude that myocardial acidosis can simulate ischemia-infarction stimulated release of bFGF. Given that pH 6.5 is a lower limit of tissue pH in vivo during myocardial ischemia, it is probable that ischemia-induced acidosis causes release of bFGF from extracellular matrix, which then may play a role in stimulating coronary collateral growth.

We extended these findings to human tissue by examining LV tissue obtained from the histologically normal heart of a 70-year-old man dying from causes not related to cardiac disease. Studies similar to the above revealed that material derived from heparin-sepharose columns eluted by NaCl gradients contained a single band of MW 14,000 contained in the 1 to 1.6 M NaCl. This protein had intense mitogenic activity on 3T3 fibroblasts and reacted by slot-blot with polyclonal antisera specific for bFGF and aFGF, suggesting that the band may be co-migrating acidic and basic FGF-like peptides, whose lower MW may reflect N- and C- terminal cleavage, or a smaller translation product characteristic of human LV myocardium.

The large animal model we are currently using to determine whether neovascularization can be induced is based on the magnitude of intracoronary collateralization that develops following implantation of the internal mammary artery (IMA) to ischemic regions of the LV. This operation has been applied to patients in the past (Vineberg opeation), but the total flow the IMA is capable of delivering is generally insufficient to importantly influence clinical outcome. We are currently implanting IMA grafts into the anterior LV wall of dogs; the dogs are randomly assigned to receive continuous administration into the IMA of either heparin or normal saline. The area of LV into which the IMA graft is placed is rendered ischemic over a 2-4 week period by positioning an ameroid constrictor around the LAD coronary artery. Animals are studied 8 weeks postoperatively to determine ischemic myocardial flow at baseline and during maximal vasodilator stimulation. Our first study demonstrated that implantation of the IMA provided nutritional myocardial flow in 50% of dogs so treated; however, the magnitude of the flow was not significantly different from control, indicating that implantation of an IMA, at least in this dog model of myocardial ischemia, is not an effective means of myocardial revascularization. The ability of heparin to promote angiogenesis in this model was then analyzed. In saline treated dogs the conductance from the IMA to the LAD circulation was linearly related to the conductance from the circumflex to the LAD beds. In other words, extrinsically derived collaterals to the LAD bed from the implanted IMA developed proportionately to the intrinsic collaterals from the circumflex to the LAD coronary arteries. This suggests an inherent propensity for collateral growth in each dog. We found that for any given intrinsic stimulus for collateral growth (as reflected in the conductance measured from the circumflex to LAD coronary arteries), conductance from the IMA to LAD beds tended to be greater in the heparin treated dogs. The differences were reflected in the larger variances in the magnitude of conductances in heparin-treated dogs, when corrected for the intrinsic propensity for collateral growth. Thus, our preliminary data suggest that collateral growth is normally con- trolled, in part, by a factor intrinsic to each animal, and that collateral formation may be responsive to heparin therapy. Studies are being initiated to further elucidate the potential for drug-induced enhancement of ischemia-induced angiogenesis.

Intravascular ablative techniques: For the past 4 years a multidisciplinary research group, coordinated through NHLBI and the Cardiology Branch, has investigated the feasability of new technologies (laser angioplasty, thermal angioplasty, and mechanical angioplasty), in an attempt to expand the range of pts with coronary and peripheral vascular disease who would be amenable to percutaneous intravascular remodeling procedures. Our approach has included initial in vitro interaction studies, atheroma "photochemistry" experiments,

small and large animal models of atherosclerosis to determine the safety and efficacy of angioplasty techniques, and prototype delivery system catheter fabrication. Human clinical trials will soon be initiated in pts with peripheral vascular disease, and later in pts with coronary artery disease.

Laser-tissue interaction studies have been performed on human necropsy tissues to determine the histologic and thermodynamic effects of varying laser sources. Superficial tissue ablation, without associated thermal tissue injury, can be optimized with a combination of proper wavelength selection (ultraviolet or infrared) and lasing parameters (pulsed versus continuous). Important developments were made in fabrication of a new Erbium:YAG laser-zirconium fluoride fiber combination. Other investigations have indicated that Excimer lasers (at 308 nm) may be somewhat disadvantageous due to a narrow laser-fiberoptic operating range, increased particulate size debris (due to shockwave generation), and less favorable acute thrombogenic endothelial surfaces. Conversely, Argon lasers, despite significant thermal tissue injury, result in preferential yellow atheroma ablation, smaller particulate size debris, and a more favorable thrombogenic surface. A pulsed visible laser system (flash-lamp pumped dye laser at 480 nm) was evaluated and appears to combine some of the favorable features of an Argon laser with micro-second pulse durations, helping to limit severe thermal tissue injury. These studies have indicated that until a more sophisticated Erbium:YAG clinical delivery system can be developed for clinical applications, it seems reasonable to proceed using a pulsed dye laser in patients with obstructive peripheral vascular disease.

Progress has been made with an industry-developed electrical thermal-tipped catheter for use in pts with peripheral vascular disease. Animal and in vitro testing suggests acceptable tissue ablation characteristics with reduced surface thrombogenicity. In addition, a catalytic thermal-tipped catheter has been invented and fabricated under the auspices of the Technical Development Branch, NHLBI. This device employs a stoicicmetric oxyhydrogen mixture and a palladium catalyst impregnated within a metallic tip to generate controlled thermal responses. Prototype devices have already been tested, using an internal thermo-couple and feedback system to monitor and regulate catheter-tip temperatures. This new device offers several advantages, including ease of fabrication, small catheter size, and precise temperature regulation, which may be important for safe and efficacious thermal angioplasty.

Extensive additional work in the area of in vitro feedback control systems has been completed and our data suggest that the fluorescence spectra signature can help identify yellow, white, hemorrhagic and calcified atheroma from normal intima and underlying normal media. Moreover, in vivo fluorescence detection in patients with and without coronary artery disease has been accomplished in 25 patients either during cardiac catheterization or coronary artery bypass graft surgery. The preliminary results from these experiments indicate that in vivo atheroma detection is feasible in a blood field despite cardiac motion and may provide the feedback signal necessary to improve target site specificity for laser angioplasty. Clinical trials using a dual laser system incorporating fluorescence plaque identification with tissue ablation in pts with peripheral vascular disease will soon commence.

## ^RONARY ARTERY DISEASE

Prognostic importance of inducible ischemia in CAD pts with resting LV dysfunction: We previously demonstrated that myocardial ischemia induced during exercise testing (defined as a decrease in LV ejection fraction and ST segment depressions on the ECG) defined a subgroup of patients with no or mild symptoms and normal LV rest function who were at high risk of dying during follow-up, and another subgroup at low risk. To assess the influence of inducible myocardial ischemia on prognosis during medical therapy of mildly symptomatic CAD pts with LV dysfunction at rest, we studied 85 consecutive pts in whom LV ejection fraction at rest ranged from 20 to 40%. We found that mortality during medical therapy was significantly associated with both the exercise ejection fraction and the magnitude of change in ejection fraction with exercise. In pts with an ejection fraction >30% the probability of survival at 6 years was 93%, compared with the survival rate of 68% in the remaining subjects. The likelihood of any cardiac event occurring was also significantly related to the magnitude of change in ejection fraction: the worst outlook was associated with decreases in exercise in ejection fraction. Of greater importance, the prognostic information related not only to pts with 3 vessel disease, but also to pts with 1 or 2 vessel disease, a subgroup which, when resting LV function is normal, has an excellent survival even in the presence of inducible ischemia. Thus, noninvasive indices of LV function and myocardial ischemia are important predictors, independent of coronary anatomy, of the clinical course of mildly symptomatic pts with LV dysfunction at rest, and may be used to identify subgroups of pts at risk of death, as well as of major cardiac events during subsequent medical therapy.

## VALVULAR HEART DISEASE

Asymptomatic aortic regurgitation: Many asymptomatic pts wtih aortic regurgitation (AR) ultimately require operation because symptoms or LV dysfunction develop. To assess the importance of serial changes in LV function in identifying such pts, we performed serial and radionuclide angiographic and echo studies in 104 asymptomatic pts with chronic AR and normal ejection fraction (EF) at rest. During follow-up of 1-13 years (mean 8 years) 3 pts died suddenly and 22 underwent surgery (18 for symptoms and 4 for subnormal EF without symptoms), an attrition rate of less than 4%/year. Multivariate analyses revealed that LV systolic dimension (LVSD) predicted outcome. When adjusted for baseline values, the average rate of change in LV systolic dimension during serial studies also predicted outcome. In pts with initial LV systolic dimension greater than 45 mm, the likelihood of death or aortic valve replacement was 9%/year. These end-points averaged 4%/year if the rate of change in LV systolic dimension was less than 1 mm/year and increased to 19% if it was greater than 1 mm/year. Thus, serial changes in LVSD helped to stratify asymptomatic pts with AR. Pts may be at low risk, despite LV dilatation, if there is no progressive change in LVSD with time.



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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04094-03 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Coronary flow reserve after dipyridamole

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


| | | |
|---|---|---|
| Richard O. Cannon, III, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| John E. Brush, M.D. | Senior Medical Staff Fellow | CB NHLBI |
| Martin B. Leon, M.D. | Co-Director 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

| 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.)


We have previously demonstrated limitation in coronary flow reserve of the coronary microcirculation to be a frequent mechanism of myocardial ischemia and angina pectoris in patients with angiographically normal epicardial coronary arteries. We have further found that limited coronary 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 ergonovine administration increases coronary resistance without discernible changes in the epicardial coronary arteries, our hypothesis is that ergonovine is inducing vasoconstriction of the coronary microcirculation, resulting in limited flow reserve to stress. Because pacing does not allow assessment of total transmural coronary flow reserve, a potent coronary arteriolar vasodilator, dipyridamole, was used to investigate peak transmural flow reserve in patients with anginal pain despite normal epicardial coronary arteries. Twenty-five patients were identified as having limited flow reserve during the stress of rapid atrial pacing following administration of ergonovine and an additional 15 patients were felt not to have evidence of coronary vasoconstriction after ergonovine administration. After administration of dipyridamole 0.5 to 0.75 mg/kg intravenously, the lowest absolute levels to which coronary resistance fell and the maximum absolute levels to which great cardiac vein flow rose were impaired in the 25 patients with ergonovine-induced flow limitation compared to the 15 patients without limitation after ergonovine administration. These studies suggest that patients with anginal chest pain despite normal epicardial coronary arteries may have exaggerated coronary responses to vasoconstrictor stimuli, which can result in myocardial ischemia during stress, as well as attenuated responses to coronary vasodilator stimuli.





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

Project Description:

We have previously found that many patients with anginal chest pain despite normal epicardial coronary arteries have a limited coronary flow response to pacing, expecially after ergonovine, associated with angina pectoris and metabolic and hemodynamic evidence of myocardial ischemia. Measurement of coronary resistance showed that ergonovine effected in increase in coronary resistance during the stress of pacing that was found in patients experiencing chest pain. Since there were only minimal changes in the epicardial coronary arteries in response to ergonovine, our hypothesis was this represent a vasoconstriction in the coronary microcirculation.

To assess whether there was also evidence of transmural limitation in coronary flow reserve, dipyridamole 0.5 - 0.75 mg/kg was infused intravenously in 40 patients following the pacing study with ergonovine. Patients who developed angina pectoris during pacing after ergonovine were considsered to have abnormal vasodilator reserve (AVR). Those without chest pain were felt to have normal flow reserve in response to pacing, even after ergonovine administration and served as controls (C). Coronary flow (CF) in ml/min was measured in the great cardiac vein by thermodilution.

| | | HR | BP | CF | CF |
|---|---|---|---|---|---|
| Baseline | C (n=15) | 77±8 | 96±11 | 72±14 | 1.37±.22 |
| | AVR (n=25) | 79±11 | 99±10 | 62±14* | 1.68±.38* |
| Pacing after ergonovine 0.15 mg/kg | C | 150±0 | 106±14 | 136±43 | .85±.27 |
| | AVR | 149±2 | 113±12* | 88±22** | 1.35±32** |
| After dipyridamole 0.5 to 0.75 mg/kg | C | 110±17 | 91±12 | 202±45 | .47±.12 |
| | AVR | 108±15 | 98±15 | 134±34** | .79±.23 |

HR=heart rate, BP=mean systemic pressure, CR=coronary resistance (BP/CF), *=p<.05, **=p<.001

In addition, 18 of the 25 patients with limted flow reserve after dipyridamole experienced chest pain despite an increase in coronary flow. In these patients, dipyridamole-induced increased flow across small prearteriolar coronary arteries narrowed because of abnormal basal vasoconstrictor tone or increased sensitivity to vasoconstrictor stimuli, could have resulted in a transmural redistribution of blood flow away from the subendocardium, precipitating subendocardial ischemia. These studies suggest that patients with anginal chest pain despite normal epicardial coronary arteries may have exaggerated coronary vascular responses to vasoconstrictor stimuli which can result in myocardial ischemia during stress, as well as attenuated responses to coronary vasodilator stimuli. Further, the site of increase resistance to coronary flow appears to be localized to pre-arteriolar small coronary arteries.

Publications:

Cannon, RO, Schenke, WH, Leon, MB, Rosing, DR, Urquhart, J, Epstein, SE: Limited coronary flow reserve after dipyridamole in patients with ergonovine-induced coronary vasoconstriction. Circulation 75: 163-174, 1987.

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04095-03 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Mechanisms 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. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Cynthia M. Tracy, M.D. | Senior Medical Staff Fellow | CB NHLBI |
| Barry J. Maron, M.D. | Senior Investigator | CB NHLBI |
| John E. Brush, M.D. | Senior Medical Staff Fellow | 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

| 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.)


We have previously demonstrated that myocardial ischemia can be induced in patients with hypertrophic cardiomyopathy by rapid atrial pacing, precipitating symptoms of chest pain and shortness of breath identical to symptoms described by history. In order to elucidate mechanisms of myocardial ischemia in patients with hypertrophic cardiomyopathy and to assess whether the presence of obstruction in left ventricular outflow mattered in the pathogenesis of ischemia, 50 patients with hypertrophic cardiomyopathy and normal epicardial coronary arteries underwent invasive study of coronary and myocardial hemodynamics in the basal state and during the stress of pacing. The 23 patients with basal obstruction (mean left ventricular outflow gradient 77±33mmHg) had significantly lower coronary resistance and higher basal coronary flow than the 27 patients without basal obstruction. During the stress of pacing, myocardial oxygen consumption and blood flow were significantly higher in patients with obstruction compared to patients without outflow obstruction. At a heart rate of 130, when most patients were experiencing chest pain, peak flow was significantly higher in patients with obstruction, with myocardial ischemia occurring at a significantly lower flow and higher coronary resistance and lower myocardial oxygen consumption in the patients without obstruction. This study suggests that the elevated left ventricular systolic pressures associated with left ventricular outflow obstruction significantly increases myocardial oxygen demands and results in rapid exhaustion of coronary flow reserve during stress. In patients without basal obstruction, exhaustion of flow reserve at a lower peak flow suggests significant impairment in coronary flow delivery.

Project No. Z01 HL 04095-03 CB

Project Description:

Fifty patients with hypertrophic cardiomyopathy underwent invasive study of coronary and myocardial hemodynamics in the basal state and during the stress of pacing. The 23 patients with basal obstruction (mean left ventricular outflow gradient, $77\pm33$mmHg; left ventricular systolic pressure of $196\pm33$mmHg, mean $\pm$ 1 standard deviation) had significantly lower coronary resistance ($.85\pm.18$ vs $1.32\pm.44$ mmHg.min/ml, $p<.001$) and higher basal coronary flow ($106\pm20$ vs $80\pm25$ ml/min, $p<.001$) in the anterior left ventricle and septum, associated with higher myocardial oxygen consumption ($12.4\pm3.6$ vs $8.9\pm3.3$ ml $O_2$/min , $p<.001$) compared to the 27 patients without obstruction (mean left ventricular systolic pressure $134\pm18$ mmHg, $p<.001$). Myocardial oxygen consumption and coronary flow were also significantly higher at paced heart rates of 100 and 130 beats/min (the anginal threshold for 41 of 50 patients) in patients with obstruction compared to those without. In patients with obstruction, transmural coronary flow reserve was exhausted at a heart rate of 130 beats/min; higher heart rates resulted in more severe metabolic evidence of ischemia with all patients experiencing chest pain, associated with an actual increase in coronary resistance. Patients without obstruction also demonstrated evidence of ischemia at heart rates of 130 and 150 beats/min, with 25 of 27 patients experiencing chest pain. In this group, myocardial ischemia occurred at significantly lower coronary flow, higher coronary resistance, and lower myocardial oxygen consumption, suggesting more severely impaired flow delivery in this group compared to those without obstruction. Abnormalities in myocardial oxygen extraction and marked elevation in filling pressures during stress were noted in both groups. Thus, obstruction in the left ventricular outflow is associated with higher left ventricular systolic pressures and oxygen consumption, and therefore has important pathogenetic importance to the precipitation of ischemia in patients with hypertrohic cardiomyopathy. Patients without obstruction may have greater impairment in coronary flow delivery, possibly related to small vessel disease.

Publications:

Cannon, RO, Schenke, WH, Maron, BJ, Tracy, CM, Leon, MB, Brush, JE, Rosing, DR, Epstein, SE: Differences in coronary flow and myocardial metabolism at rest and during pacing between patients with obstructive and patients with non-obstructive hypertrophic cardiomyopathy. J Am Coll Cardiol, 1987. (In press, July issue).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04096-03 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Charles L. McIntosh, M.D. | Senior Surgeon | SB 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, Betheda, Md

| 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.)

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, 20 patients were studied at rest and during atrial pacing before and after operation (septal myectomy in 14 and mitral valve replacement in 6). Coronary flow to the anterior left ventricle and septum, the site of maximum hypertrophy in these patients, was assessed by thermodilution. In all 20 patients there was successful relief of resting left ventricular outflow tract gradient from a preoperative gradient of $61 \pm 35$ to $4 \pm 7$ mmHg postoperatively. Surgical relief of left ventricular outflow tract obstruction significantly 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 anginal threshold and metabolic evidence of ischemia. These results demonstrate the importance of left ventricular outflow tract gradients in hypertrophic cardiomyopathy as well as the mechanism of improved effort tolerance after surgical relief of obstruction.

Project Descripion:

To determine the effects of surgical relief (septal myotomy/myectomy or mitral valve replacement) of left ventricular outflow tract obstruction in patients with hypertrophic cardiomyopathy, 20 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. Values = mean ±1 S.D.

Before Operation — gradient = 61±35

| | BP | CF | LVSP | MV02 | Lact+ |
|---|---|---|---|---|---|
| Rest | 90±16 | 99±25 | 182±30 | 11.7±3.9 | |
| Pacing | 106±17 | 158±41 | 160±25 | 19.0±6.0 | 13/20 |

After operation

| | BP | CF | LVSP | MV02 | Lact+ |
|---|---|---|---|---|---|
| Rest | 91±16 | 78±16* | 128±22* | 8.5±1.9* | |
| Pacing | 96±20* | 134±46 | 122±20* | 14.6±5.6** | 6/20 |

* = p<.05, **= p<.01 vs respective preoperative value.
BP= mean systemic pressure (mmHg), CR= coronary flow (ml/min), LVSP= left ventricular systolic pressure (mmHg), MV02 = myocardial oxygen consumption (ml 02/min), lact+ = lactate production (evidence for anaerobic metabolism).

All patients developed angina at a heart rate of 110-130 preoperatively; 14 out of 20 patients improved their anginal threshold postoperatively by at least 20 beats/min, with 6 patients experiencing no chest pain during pacing after operation. Left ventricular end-diastolic pressure rose to 31±7 preoperatively and to 22±7 postoperatively (p<.001). 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 as well as the mechanism of relief of ischemia and improvement in symptoms following surgical relief of outflow obstruction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04109-03 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Promotion of angiogenesis by heparin in the canine heart

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

| | | | |
|---|---|---|---|
| Ellis F. Unger, M.D. | Senior Staff Fellow | CB | NHLBI |
| Cedric D. Sheffield, M.D. | Staff Fellow | CB | NHLBI |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB | NHLBI |

COOPERATING UNITS *(if any)*

Veterinary Resources Branch, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: 1.2 | PROFESSIONAL: 0.7 | 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 purpose of this investigation, completed in April, 1987, was to assess the ability of heparin to promote angiogenesis in a canine model of myocardial ischemia. Ameroid devices were placed on the proximal left anterior descending coronary arteries (LAD) of 32 dogs, causing progressive LAD occlusion over a 2-3 week period. The left internal mammary artery (IMA) was implanted intramyocardially in the LAD zone, with the hope that collaterals would develop from the IMA to the LAD. Dogs received continuous infusions directly into the IMA starting the day of surgery and continuing for 8 weeks. Dogs were randomized to 3 treatment groups: heparin, 15 units/hr (group 1); heparin, 15 units/hr for 2 weeks, followed by 150 units/hr for 6 weeks (group 2); or saline (group 3). After 8 weeks, IMA to LAD conductance and left circumflex coronary artery (LCX) to LAD conductances were assessed during adenosine-induced vasodilation using the microsphere technique. In saline treated dogs, conductance from the IMA to the LAD was linearly related to conductance from the LCX to the LAD ($R = 0.77$, p less than 0.05). Thus, collaterals from both the implanted artery and the native coronary artery to the LAD area developed proportionately. Although not statistically significant, there was a tendency towards higher IMA to LAD conductance in heparin treated dogs. Thus the data suggest that the propensity for coronary collateral growth may be related to factor(s) intrinsic to each animal, and that collateral formation may be responsive to heparin. We hope that more potent interventions can more significantly enhance collateral development, and we are directing future efforts to study this possibility.

Project No. Z01 HL 04109-03 CB

Project Description:

The ability of heparin to promote angiogenesis was assessed in a canine model of myocardial ischemia. Ameroids were placed on the proximal LAD of 32 dogs. The left internal mammary artery (IMA) was implanted intramyocardially in the LAD zone. Dogs randomly received continuous infusions directly into the IMA: Heparin, 15 units/hr (group 1), Heparin, 15 units/hr for 14 days, followed by 150 units/hr (group 2), or saline (group 3). After 8 weeks, conductances from IMA to LAD (C-IL) and LCX to LAD (C-CL) were assessed during adenosine using microspheres. Conductance is reported as ml/min/100g/mmHg +/- SEM.

| GROUP | N | IMA to LAD CONDUCTANCE | LCX to LAD CONDUCTANCE |
|---|---|---|---|
| 1 | 10 | 0.64 +/- 0.18 | 2.44 +/- 0.76 |
| 2 | 8 | 0.73 +/- 0.37 | 2.53 +/- 0.48 |
| 3 | 14 | 0.45 +/- 0.15 | 2.66 +/- 0.28 |

In saline treated dogs, C-IL was linearly related to C-CL ($R=.77$, $p<.05$). Thus IMA to LAD and LCX to LAD collaterals developed proportionately, suggesting an inherent propensity for collateral growth in each dog. For any given intrinsic stimulus (reflected by C-CL), C-IL tended to be greater in the H treated dogs. The differences were reflected in the variance of C-IL/C-CL ratios (0.05 vs 4.53; saline vs H; $p<.09$). Thus, the data suggest that collateral growth is normally controlled by a factor intrinsic to each animal, and that collateral formation may be responsive to heparin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04111-03 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Coronary flow reserve in idiopathic dilated cardiomyopathy

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

| | | |
|---|---|---|
| Richard O. Cannon, III, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Robert E. Cunnion, M.D. | Senior Staff Fellow | CCMD CC |
| Joseph E. Parrillo, M.D. | Head, Critical Care Medicine Dept. | CCMD CC |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB NHLBI |

COOPERATING UNITS (if any)

Critical Care Medicine Dept., Clinical Center, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)


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 epicardial coronary arteries. To examine whether abnormalities in coronary flow exist in dilated cardiomyopathy, 26 patients with dilated cardiomyopathy and normal epicardial coronary arteries, 12 of whom had frequent chest pain by history, underwent measurement of great cardiac vein flow and myocardial metabolism at rest and during pacing to a heart rate of 150. During pacing following administration of ergonovine, all 12 patients with a history of chest pain experienced their typical pain. Compared to patients withut chest pain, their coronary flow was lower and coronary resistance higher, with increased myocardial oxygen extraction suggestive of myocardial ischemia. Additionally, there was a greater increase in left ventricular filling pressures 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 20 patients demonstrated that those 7 patients with a history of angina pectoris also had impairment in transmural coronary flow reserve compared to the 13 patients without chest pain. Thus, patients with dilated cardiomyopathy and chest pain by history may have limited coronary vasodilator reserve, expecially after vasoconstrictor stimulus. Whether this contributes to myocardial damage in dilated cardiomyopathy or is an epiphenomenon of an unrelated etiology, remains to be determined.




PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Twenty-six patients with dilated cardiomyopathy and angiographically normal coronary arteries, 12 of whom gave a history of anginal chest pain, underwent non-invasive and invasive hemodynamic study. During exercise treadmill testing, patients with a history of angina demonstrated worse effort tolerance (7'25" ± 4'53" vs 13'59" ± 5'09", $p<0.005$) and a lower end-exercise systolic blood pressure-heart rate product (17.9 ± 3.4 vs 23.6 ± 4.9 mm Hg.beats/minute x $10^3$, $p<0.005$) compared to patients without a history of angina. During rapid atrial pacing after ergonovine 0.15 mg intravenously, 11 of 12 patients with a history of angina experienced their typical chest pain, in contrast to only 1 of 14 patients without a history of angina. The angina group, compared to the non-angina group, had significantly lower great cardiac vein flow (118 ± 24 vs 160 ± 43 ml/minute, $p<0.01$), and higher coronary resistance (0.87 ± 0.21 vs 0.66 ± 0.25 mm Hg.minute/ml, $p<0.05$), significant widening of the arterial minus great cardiac vein oxygen difference, and a significant fall in cardiac index during pacing. Further, ergonovine resulted in higher coronary resistance during pacing in the angina group compared to pacing alone (+ 0.16 ± 0.16 mmHg minute/ml, $p<0.01$), in the absence of significant reduction in epicardial coronary artery luminal diameter. After dipyridamole 0.5-0.75 mg/kg intravenously to 20 patients, the 7 with a history of angina had significantly lower flow (149 ± 37 vs 218 ± 73 ml/minute, $p<0.05$) and higher coronary resistance (0.59 ± 0.09 vs 0.43 ± 0.17 mm Hg.minute/ml, $p<0.05$) than the non-angina group. We conclude that patients with dilated cardiomyopathy and chest pain unrelated to epicardial coronary artery disease exhibit impaired vasodilator responses to both metabolic and pharmacologic stimuli, and an increased sensitivity to the vasoconstrictor effects of ergonovine. Whether these findings are of etiologic or long-term prognostic significance, are as yet unknown.

Publications:

Cannon, RO, Cunnion, RE, Parrillo, JE, Palmeri, ST, Tucker, EE, Schenke, WH, Epstein SE: Dynamic limitation of coronary vasodilator reserve in patients with dilated cardiomyopathy and chest pain. J Am Coll Cardiol 1987, (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04112-02 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Quantitation of Calcium Channels in Human Myocardium

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


| | | |
|---|---|---|
| Frederic L. Sax, M.D. | Medical Staff Fellow | CB NHLBI |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB NHLBI |
| Charles McIntosh, M.D. | Senior Surgeon | CB NHLBI |
| William Roberts, M.D. | Chief, Pathology Service | PA NHLBI |


COOPERATING UNITS (if any)

Cardiac Surgery, Pathology, NIH
Johns Hopkins University

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .4 | .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.)


Clinical evidence points to the possibility that patients with hypertrophic cardiomyopathy (HCM) may have disordered regulation of cytosolic calcium. We hypothesized that these patients might have an increased number of calcium channels so that for a given signal, they have increased calcium influx. Such an increased number of calcium channels has recently been reported in the Syrian hamster model of cardiomyopathy. To study calcium channel density in human myocardium we have been using right atrial appendages isolated during cardiac surgery on patients with or without HCM.

We now have evidence to suggest that patients with hypertrophic cardiomyopathy have elevated levels of Ca+2 antagonist binding sites (thought to be equivalent of calcium channels) compared to other patients undergoing cardiac surgery. This increase appears to be selective as there is no increase in sodium-fast channels or B-receptors. The levels of Ca+2 antagonist binding sites in the right atrial appendage correlate well with those in the septum of patients with HCM. These findings suggest that an increased number of calcium channels may play an important role in the pathophysiology of this disease.




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

Project No. Z01 HL 04112-02 CB

Project Description:

Calcium channel density in human myocardium is being studied in patients with hypertrophic cardiomyopathy (HCM) and other cardiac patients undergoing open heart surgery requiring cardiopulmonary bypass. The purpose of this is to determine if patients with HCM have a higher density of calcium channels than a "control" population.

Tissue removed as a routine part of cardiopulmonary bypass, namely the right atrial appendage, is frozen on dry ice after being weighed and recorded in Surgical Pathology. It is stored at $-70^{o}C$ until ready for study.

The tissue is then transported to our collaborators at Johns Hopkins where it is processed as follows: The tissue is homogenized in buffer, filtered and centrifuged to form membrane preparations. These preparations are incubated with tritiated-nitrendipine (which binds specifically to a receptor located in or near the Ca+2 channel) in the presence or absence of unlabeled nitrendipine. This allows one to determine specific binding to calcium channels and quantitate calcium channel density.

Abnormal adrenergic stimulation and abnormal calcium regulation may play a role in the pathogenesis of hypertrophic cardiomyopathy (HCM). For this reason, we studied the density of beta-adrenoceptors and calcium antagonist receptors in right atrial appendages of HCM patients and patients with other cardiac disorders undergoing cardiac surgery.

Eleven HCM and 10 non-HCM appendages were studied using [$^{3}H$] PN200-110 in a ligand binding assay. HCM patients displayed a greater number of dihydropyridine calcium antagonist binding sites than non-HCM patients ($374 \pm 100$ vs $261 \pm 61$ fmole/mg protein, mean$\pm$SD, $p<0.005$). Tissue from 6 HCM patients and 13 non-HCM patients was also assayed using [$^{125}I$] iodocyanopindolol. The density of beta-adrenoceptors was not different between the groups ($121 \pm 76$ vs $108 \pm 82$, although the density of beta-adrenoceptors was higher in pts receiving beta-antagonist medication then those not receiving these drugs ($165 \pm 86$ vs $89 \pm 60$, $p<0.05$).

These results suggest that calcium antagonist receptors, and thus calcium channels, are selectively elevated in the myocardium of HCM patients and that abnormal clcium fluxes may be important in the pathophysiology of this disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04113-02 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Platelet calcium levels in hypertrophic cardiomyopathy

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


| | | |
|---|---|---|
| Frederic L. Sax, M.D. | Medical Staff Fellow | CB NHLBI |
| Antonio Bartorelli, M.D. | Visiting Researcher | CB NHLBI |
| Michael A. Beaven, Ph.D. | Senior Investigator | CP NHLBI |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB NHLBI |


COOPERATING UNITS (if any)

Laboratory of Chemical Pharmacology

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .3 | .2 | .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.)


There is circumstantial clinical evidence to suggest that patients with hypertrophic cardiomyopathy (HCM) have disordered regulation of intra-cellular calcium. The hypercontractile myocardium with poor diastolic relaxation could be explained, for example, by increased cytosolic Ca+2 in the cardiac myocyte. Given that some forms of HCM are genetically transmitted, we postulated that a disorder of cytosolic calcium metabolism might be present in other, non-cardiac tissue. To study this, we isolated platelets from the plasma of patients with HCM and normals and measured intra-cellular calcium levels using the fluorescent indicator QuinII. Our preliminary results (on only a small number of patients and controls) indicates that resting Ca+2 levels are the same in these populations. When the cells are stimulated with vasopressin --which causes both intra-cellular calcium mobilization and Ca+2 influx -- some patients appear to have a blunted response to this stimulant. Whether this is an epiphenomenon still remains to be determined, but if these results are substantiated, they point to wide-spread Ca+2 dysregulation. The mechanism of this could be determined and might give us an important clue as to the etiology of this disease.




PHS 6040 (Rev. 1/84) GPO 914-913

Project Description

Platelet calcium levels are being studied in patients with hypertrophic cardiomyopathy and normal age and sex-matched controls. The purpose of this is to look for evidence of cytosolic calcium dysregulation in patients with HCM.

Twenty to thirty cc of blood are withdrawn antecubitally from participating subjects, placed in citrated plastic tubes and centrifuged to obtain platelet-rich plasma. This plasma is incubated with Quin-II ester, which, on being taken up by the cells, is hydrolyzed to Quin-II which fluoresces in the presence of calcium. The platelets are then separated from the plasma and excess Quin-II on a sepharose IIB column and studied in a suspension of Tyrodes solution to which calcium is restored. Platelet Ca+2 regulatory mechanisms are studied by stimulation of calcium mobilization and influx by vasopressin and platelet-activating factor (both of which stimulate phosphotidylinositol (PI) metabolism.).

Preliminary results with Quin-II pointed to comparable resting valves of intra-cellular calcium between patients with hypertrophic cardiomyopathy and normal controls. The hypertrophic patients (5/7 in a preliminary series) appeared to have a blunted response to stimulation, however. Since we were concerned that this effect may in part be due to a buffering effect of Quin-II, we recently switched our assay to use FuRA-2 and ratiometric measurements. This work is currently in progress.

If substantiated, further work will need to be done to determine the locus of the abnormality (PI metabolism, Ca+2 reuptake/extrusion, etc). Furthermore, modulating factors such as stimulants of adenylate cyclase (forsokolin), protein kinase C (phorbol esters) and growth factors will be studied to determine their possible contribution.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04114-02 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Forearm flow in patients with angina and normal coronary arteries

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


| | | |
|---|---|---|
| Frederic L. Sax, M.D. | Medical Staff Fellow | CB NHLBI |
| Richard O. Cannon, III, M.D. | Co-Director Cardiov. 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

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .3 | .15 | .15 |

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 anginal chest pain and normal epicardial coronary arteries show abnormal coronary flow response to electrical pacing, the vasoconstrictor, ergonovine and the vasodilator, dipyridamole. These stimuli, in fact, often reproduce their chest pain. The studies eliciting these data suggest these patients have dysregulation of vascular smooth muscle tone, and, in particular, decreased vasodilator reserve. To examine if these phenomena represent a more generalized abnormality of vascular smooth muscle function, we studied another vascular bed by studying blood flow to skeletal muscle in the forearm. We used the non-invasive technique of strain-gauge plethysmography and studied vasodilator capacity by subjecting the forearm to ischemia (an upper arm cuff inflated to supra-systolic pressures) of increased lengths of duration. Compared to a control population of comparable age and sex, the patients with microvascular angina had blunted peak flows at all durations of ischemic time (1 min, 3 min, 5 min, 10 min). Their minimal vascular resistance (mean blood pressure divided flow) was also higher than controls. This suggests a decreased vasodilator capacity of the forearm musculature and points to a more generalized disorder of smooth muscle function in these patients.

Project No. Z01 HL 04114-02 CB

Project Description:

Many patients with anginal chest pain and normal epicardial arteries exhibit a reduced myocardial flow response to pacing which is exacerbated by ergonovine. They also have decreased vasodilator reserve to dipyridamole. These data suggest a dynamic abnormality of small coronary artery tone.

To determine if a more generalized vascular abnormality might be present, we studied forearm blood flow and the hyperemic response to forearm ischemia using strain gauge plethysmography. This hyperemic response was used as an index of vasodilator capacity in the forearm skeletal muscle vasculature.

Sixteen patients and 16 controls were studied. There were no statistically significant differences between the patients and controls in age, mean arterial blood pressure, rest flow, or rest vascular resistance. Peak flows after forearm ischemia were as follows (mean $\pm$ S.D.):

| Time of Ischemia | Patients | Controls | |
|---|---|---|---|
| 1 min | 16.1$\pm$6.0 | 1.50$\pm$1.8 | N.S. |
| 3 min | 26.7$\pm$8.6 | 36.3$\pm$8.0 | $p<0.02$ |
| 5 min | 24.8$\pm$7.7 | 37.8$\pm$5.7 | $p<0.02$ |
| 10 min | 31.7$\pm$10.5 | 39.9$\pm$5.0 | $p<0.02$ |

Vascular resistance after 10' of ischemia was 3.3$\pm$1.0 in patients, 2.2$\pm$0.3 controls ($p<.003$). There was also a strong correlation between minimum resistance measured in the forearm of patients with microvascular angina and minimum resistance after dipyridamole in the coronary bed ($r=0.74$, $p<0.005$).

These findings suggest that patients with angina, normal epicardial coronary arteries, and abnormal coronary vasodilator reserve have a more generalized smooth muscle disorder characterized by impaired vasodilator capacity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04115-02 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Forearm flow in patients with hypertrophic cardiomyopathy

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


| | | |
|---|---|---|
| Frederic L. Sax, M.D. | Medical Staff Fellow | CB NHLBI |
| James E. Udelson, M.D. | Medical Staff Fellow | 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

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .5 | .25 | .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.)


Patients with hypertrophic cardiomyopathy (HCM) exhibit abnormalities in coronary flow response to stress stimuli such as pacing and ergonovine, and the vasodilator, dipyridamole. These stimuli often elicit anginal chest pain in such patients. It has therefore been suggested that patients with HCM have decreased small coronary vasodilator reserve. To determine if this phenomenon applies to other vascular beds, we studied the forearm vasodilator capacity using ischemia (occlusion of the circulation) as the vasodilator stimulus. We found that patients with HCM in fact do have decreased vasodilator capacity in their forearm vasculature compared to normals. This is manifested by a decreased peak flow and increased vascular resistance at various durations of ischemia, compared to normals. This suggests that patients with HCM may have an abnormality of smooth muscle regulation that affects both the myocardial and peripheral vasculature.




PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Patients with hypertrophic cardiomyopathy (HCM) exhibit abnormalities in coronary flow responses to ergonovine and dipyridamole, suggesting an impairment of coronary vasodilator reserve. This impairment may be due to hypertrophy of the vessel walls in the coronary vasculature, or it may reflect a more dynamic functional abnormality of vascular smooth muscle tone.

To determine whether HCM patients have a more generalized disorder of vascular smooth muscle vasodilator reserve, we used mercury-in-silastic strain gauge plethysmography to study the hyperemic response to forearm ischemia. We excluded patients with outflow tract obstruction, hypertension, elevated filling pressures or CHF symptoms. Only patients who were NYHA functional class I or II (for chest pain) were used. This was done to avoid known confounding variables that affect forearm vasodilation after ischemia.

The age, sex, mean arterial blood pressure, resting forearm flow and rest vascular resistance of the 21 hypertrophic cardiomyopathy patients were no different than that of the 16 normal controls. Peak flows after ischemia were as follows (mean ± S.D.):

| Time of ischemia | HCM | Controls | |
|---|---|---|---|
| 1' | 12.1±3.9 | 17.8±3.6 | $p<.0001$ |
| 3' | 24.2±5.4 | 36.3±8.0 | $p<.0001$ |
| 5' | 28.7±5.6 | 37.8±5.7 | $p<.0001$ |
| 10' | 33.1±5.7 | 39.9±5.0 | $p<.001$ |

Vascular resistance after 10' ischemia was 2.8±0.7 hypertrophic cardiomyopathy and 2.2±0.3 in controls ($p<0.01$).

These findings suggest that patients with hypertrophic cardiomyopathy may have a generalized disorder of vascular smooth muscle vasodilator capacity, affecting both the peripheral and myocardial vasculature. Further work will be needed to determine if this is a primary abnormality of smooth muscle or a secondary response (i.e. via a neurohumeral axis) to the patients underlying heart disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04127-02 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Abnormal esophageal motility in patients with limited coronary flow reserve

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

| | | |
|---|---|---|
| Richard O. Cannon, III, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Edward L. Cattau, M.D. | Gastroenterology Div.-Georgetown Univ. Hosp. | |
| Stanley B. Benjamin, M.D. | " " " " " | |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB NHLBI |

COOPERATING UNITS (if any)

Georgetown University Hospital

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .1 | .1 | |

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.)

Explanations of chest pain in patients with chest pain, despite angiographically normal coronary arteries include abnormal coronary flow reserve and esophageal motility disorders. To ascertain the frequency of cardiac versus esophageal functional abnormalities in such patients, 50 patients with chest pain despite normal epicardial coronary arteries underwent measurement of coronary resistance during pacing at a heart rate of 150, heart rate of 150 after ergonovine, 0.5-0.3 mg intravenously and after dipyridamole 0.5-0.75 mg/kg intravenously. Those patients who had dynamic limitation in flow reserve to ergonovine and limited flow reserve after dipyridamole had a higher prevalence of esophageal motility disorders than those patients who had no vasoconstrictor response to ergonovine and had a normal flow reserve after dipyridamole. The high prevalence of abnormal esophageal motility in patients with dynamic limitation in coronary flow reserve suggests that this syndrome may be part of a generalized abnormality of smooth muscle reactivity.

Project Description:

Explanations for chest pain in patients with anginal pain despite angiographically normal coronary arteries include abnormal coronary flow reserve and esophageal motility disorders. To ascertain the frequency of cardiac versus esophageal function abnormalities in such patients, 50 patients with chest pain despite normal coronary arteries underwent measurement of coronary resistance during pacing at a heart rate of 150, heart rate of 150 after ergonovine 0.5-0.3 mg and after dipyridamole 0.5-.75 mg/kg to achieve maximum pharmacologic flow reserve. Group A patients had an increase in coronary resistance during pacing after ergonovine, with most experiencing chest pain. Group N patients had no vasoconstrictor response to ergonovine. No patient had epicardial coronary artery spasm after ergonovine. Group A also had a higher minimal coronary resistance after dipyridamole.

| | CR | CR-E | CP | CR-D |
|---|---|---|---|---|
| Group A (n=38) | 1.06±.33 | 1.32±.36 | 34/38 | .65±.21 |
| Group N (n=12) | .84±.18 | .77±.12 | 1/12 | .46±.09 |

CR=coronary resistance (mean systemic pressure / great cardiac vein flow) mmHg.min/ml
CR-E = coronary resistance following ergonovine administration
CP = chest pain
CR-D = coronary resistance following dipyridamole administration
*=p<.05, +=p<.01 vs Group A

All underwent esophageal motility testing. Criteria for high amplitude peristalsis were met by 22 of 38 (58%) group A patients compared to 4 of 12 (33%) group N patients (p=.06). The high prevalence of abnormal esophageal motility in patients with dynamic limitation in coronary flow reserve suggests that this syndrome may be part of a generalized abnormality of smooth muscle reactivity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04129-02 CB

PERIOD COVERED

October 1, 1986 to September 30, 1986

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

Amiodarone therapy in patients with HCM and refractory cardiac symptoms

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

| | | |
|---|---|---|
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Robert O. Bonow, M.D. | Chief, Nuclear Cardiology | CB NHLBI |
| Cynthia M. Tracy, M.D. | Senior Medical Staff Fellow | 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

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.2 | 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.)

An ongoing clinical trial has been formulated to assess the therapeutic efficacy of oral amiodarone in patients with hypertrophic cardiomyopathy and either refractory cardiac symptoms or patients who are at high risk for sudden cardiac death. Over the past year, we have completed a study which now includes 72 patients with hypertrophic cardiomyopathy ($41 \pm 14$ years) as part of two protocols: Group 1 - patients with refractory cardiac symptoms (n=50) and Group 2 - patients who are at high risk for sudden death (n=22). Despite improved symptoms in Group 1 patients, and reduced ventricular arrhythmias in Group 1 and 2 patients, during the follow-up period ($662 \pm 273$ days), 8 patients had sudden cardiac death (cummulative survival=88%). Sudden death appeared to occur early after instituting amiodarone treatment in patients with severe cardiac symptoms, and later in patients with high risk variables for sudden cardiac death. Unfortunately, we conclude that amiodarone does not prevent and may increase sudden cardiac death in certain subsets of patients with hypertrohic cardiomyopathy.



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

Project Description:

Others have indicated that in pts with hypertrophic cardiomyopathy (HCM) and nonsustained ventricular tachycardia (NSVT), amiodarone (A) may improve survival (S) by preventing sudden death (SD). At the NIH, 72 HCM pts ($41 \pm 14$ yrs) were treated with A (maintenance dose $451 \pm 128$mg) and followed as part of 2 study protocols: Group 1 (n=50)-pts with refractory cardiac symptoms (Sx), Group 2 (n=22)-pts at high risk for SD. Baseline characteristics for the 2 groups were similar except for functional class (1=3.3 vs 2=2.0, $p<.001$) radionuclide peak filling rate (PFR) (1=2.9 vs 2=3.9, $p<.005$), and Holter NSVT (1=36% vs 2= 73%, $p<.01$). Despite improved Sx in Group 1 and reduced Holter NSVT (from 47% to 7%, $p<.001$) in Groups 1+2, 8 pts had SD (cumulative S=88%) during the follow-up period ($662 \pm 273$ days). Importantly, SD was early (<6 mos) in all Group 1 pts (6/50) and in 2 pts was associated with new Holter NSVT on A. In Group 1 pts, an early fall in PFR during A predicted subsequent SD events ($p< .001$). In Group 2 pts, SD occurred later (>6mos), was less frequent (2/22), and was unrelated to changes in Holter or radionuclide variables. We conclude that in severely symptomatic HCM pts, A does not prevent, and may increase SD, presumably by a proarrhythmic action that is possibly potentiated by a deterioration in diastolic function.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04130-02 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

A new erbium laser and infrared fiber system for laser angioplasty

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

| | | |
|---|---|---|
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Louis G. Prevosti, M.D. | Research Associate | CB NHLBI |
| Robert F. Bonner, Ph.D. | Senior Research Fellow | BEIB DRS |
| Paul D. Smith, Ph.D. | Senior Research Fellow | BEIB DRS |

COOPERATING UNITS (if any)

Naval Research Laboratory
Quantronics Laser Co. Infrared Fibers Systems

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.2 | 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.)

This is a continuation of previous research experiments attempting to characterize the optimal laser source and transmitting optical fibers for intravascular precise microablative surgery. Over the past year, considerable additional work has helped to characterize the optimal lasing parameters for variable composition plaque ablation. In addition, substantive progress has been made in developing zirconium fluoride glass fibers to meet power transmission, flexibility, strength, and durability requirements. Catheter and fiber tip development is proceeding such that we have demonstrated high efficiency ablation of tissue in a wet field through flexible catheters using a 200 micron fiber filling a 1.5mm sapphire output window. These preliminary studies have demonstrated the feasibility of Er:YAG laser and zirconium fluoride fibers as a system for laser angioplasty.



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

Project Description:

Pulsed infrared Er:YAG laser light at 2.9u provides the strongest tissue absorption (~10,000 $cm^{-1}$) compatiable with optical fiber transmission. A pulse (200 usec) of laser light at 2.9u is absorbed at the surface leading to the rapid sequential vaporization of 1u-thick layers of atheroma (including heavily calcified) for depths (up to several hundred u) proportional to pulse fluence >20 $mJ/mm^2$. The resulting surface is smoothly etched (within 5u) showing minimal thermal damage (<10u) and reduced platelet deposition; the particulate debris was mostly submicroscopic. Thus the Er:YAG laser may be an ideal choice for laser angioplasty.

A critical factor in the clinical application of this pulsed infrared laser has been the refinement of zirconium fluoride (ZrF) glass fibers to meet power transmission, flexibility, strength and durability requirements. The properties of prototype commercial fibers of high strength (~100 kpsi), flexibility (6mm bend radius), damage threshold (>9$J/mm^2$), and transmission (~2% loss per m) were measured. These properties approach those of silica fibers used with conventional lasers. High-efficiency ablation of tissue in a wet field through flexible catheters using a 200u ZrF fiber filling a 1.5mm sapphire output window demonstrates the feasibility of this system for laser angioplasty.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04138-02 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Influence of age on left ventricular diastolic function

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

| | | |
|---|---|---|
| Paolo Spirito, M.D. | Guest Worker | CB NHLBI |
| Barry J. Maron, M.D. | Senior Investigator | 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: |
|---|---|---|
| 0.7 | 0.5 | 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 present investigation was performed to assess the influence of age on Doppler variables of left ventricular diastolic function. Six Doppler diastolic indexes were analyzed in 86 normal volunteers ranging in age from 20 to 74 years (mean 36). All 6 indexes sowed a linear relationship to age. Duration of isovolumic relaxation, duration of the early diastolic flow-velocity peak and maximal late diastolic (atrial) flow-velocity increased with age ($r=0.41$, $r=0.42$, and $r=0.63$, respectively; $p<0.01$ to $p<0.001$). Conversely, maximal early diastolic flow-velocity, the rate of decrease (descent) of flow-velocity in early diastole, and the ratio between maximal early and late diastolic flow-velocities decreased with age ($r= -0.40$, $r= -0.42$, and $r= -0.66$, respectively; $p<0.001$). Comparison of Doppler indexes of diastolic function among different age groups (20 to 29 years, 30 to 49 years, and 50 to 74 years) also demonstrated an influence of age on these diastolic variables. Isovolumic relaxation was significantly prolonged in older subjects compared to either the intermediate ($p<0.05$) or the younger age groups ($p<0.001$). In addition, both the rate of decrease of flow-velocity in early diastole and the ratio between maximal early and late diastolic flow-velocities were reduced in older compared to younger subjects ($p<0.001$). In conclusion, the isovolumic relaxation phase and the early and late filling phases of diastole, as assessed by Doppler echocardiography, appear to be importantly affected by aging. Specifically, in older subjects, the duration of isovolumic relaxation is prolonged and the rate of early diastolic filling velocity is reduced: as an apparent compensation, the relative contribution of atrial systole to overall left ventricular filling is increased. These diastolic alterations qualitatively resemble those observed in patients with cardiac diseases associated with left ventricular diastolic impairment; hence, the effects of age should be taken into consideration in formulating "normal" limits for left ventricular diastolic function.



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

Project No. Z01 HL 04138-02 CB

Project Description:

Recently, Doppler echocardiography has been utilized to assess left ventricular diastolic function in patients with a variety of cardiac diseases and in normal subjects. However, some determinants of left ventricular filling assessed with Doppler would appear to be affected by age. Such age-related changes in left ventricular filling patterns may be potentially important in defining normal values for diastolic function. The present investigation was performed to assess the influence of age on Doppler variables of left ventricular diastolic function. Six Doppler diastolic indexes were analyzed in 86 normal volunteers ranging in age from 20 to 74 years (mean 36). All 6 indexes showed a linear relationship to age. Duration of isovolumic relaxation, duration of the early diastolic flow-velocity peak and maximal late diastolic (atrial) flow-velocity increased with age ($r = 0.41$, $r = 0.32$, and $r = 0.63$, respectively; $p<0.01$ to $p<0.001$). Conversely, maximal early diastolic flow-velocity, the rate of decrease (descent) of flow-velocity in early diastole, and the ratio between maximal early and late diastolic flow- velocities decreased with age ($r = -0.40$, $r = -0.42$, and $r = -0.66$, respectively; $p<0.001$).

Comparison of Doppler indexes of diastolic function among different age groups (20 to 29 years, 30 to 49 years, and 50 to 74 years) also demonstrated an influence of age on these diastolic variables. Isovolumic relaxation was significantly prolonged in older subjects compared to either the intermediate ($p<0.05$) or the younger age groups ($p<0.001$). In addition, both the rate of decrease of flow-velocity in early diastole and the ratio between maximal early and late diastolic flow-velocities were reduced in older compared to younger subjects ($p<0.001$).

In conclusion, the isovolumic relaxation phase and the early and late filling phases of diastole, as assessed by Doppler echocardiography, appear to be importantly affected by aging. Specifically, in older subjects, the duration of isovolumic relaxation is prolonged and the rate of early diastolic filling velocity is reduced; as an apparent compensation, the relative contribution of atrial systole to overall left ventricular filling is increased. These diastolic alterations qualitatively resemble those observed in patients with cardiac diseases associated with left ventricular diastolic impairment; hence, the effects of age should be taken into consideration in formulating "normal" limits for left ventricular diastolic function.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04144-02 CB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Reproducibility of Doppler echocardiographic measurements of diastolic function

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


| | | |
|---|---|---|
| Paolo Spirito, M.D. | Guest Researcher | CB NHLBI |
| Barry J. Maron, M.D. | Senior Investigator | CB NHLBI |
| Joel I. Verter, Ph.D. | Biostatistician | CB NHLBI |


COOPERATING UNITS (if any)
Division of Epidemiology and Clinical Applications, Biostatistics Research Branch.

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.6 | 0.5 | 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 present investigation was undertaken in 12 normal subjects to determine the magnitude of technical and biologic variability of six previously validated Doppler indexes of diastolic function. Technical variability due to the reader was small for each of the six Doppler indexes. Variability due to the technician was larger than for the reader, and became sizeable when measurements obtained in a single subject were compared; however, variability due to the technician was negligible when mean differences between groups of subjects were analyzed, and none of these differences achieved statistical significances. Day-to-day variability (i.e., biologic variability) was larger than technical variability (reader and technician) for the great majority of the comparisons, both in the individual (28 of 30 comparisons) and group data analyses (27 of 30 comparisons). The two Doppler indexes assessing late diastolic events (i.e., maximal late diastolic [atrial] flow-velocity, and the ratio between maximal early and late flow velocity) did show significant change on a day-to-day basis ($p<0.05$). In conclusion, Doppler indexes that represent a measure of the relaxation and early filling phases of diastole showed good reproducibility for group data analysis. Hence, these Doppler indexes can be utilized to assess left ventricular diastolic function noninvasively in large groups of patients. However, extensively utilized indexes assessing the atrial contribution to ventricular filling, such as maximal late (atrial) diastolic flow-velocity and the ratio between maximal early and late flow-velocities, have a relatively large variability for both individual and group data analysis; therefore, conclusions based on these late diastolic indexes should be drawn with caution.

Project No. Z01 HL 04144-02 CB

Project Description:

Recently, pulsed Doppler echocardiography has been utilized to assess left ventricular diastolic performance in patients with cardiac disease. Indexes of diastolic function derived from the Doppler diastolic flow-velocity waveform have been shown to correlate well with indexes of left ventricular filling determined by either contrast angiography or radionuclide angiography, and also with measures of isovolumic relaxation derived from digitized M-mode echocardiography.

The noninvasive nature of Doppler echocardiography would make this technique a particularly useful tool for the assessment of left ventricular diastolic performance in large group of patients and for the serial evaluation of the effects on diastolic function of medical or surgical interventions. However, before data obtained from the Doppler diastolic waveform can be utilized for longitudinal and cross-sectional studies of diastolic function in patient with cardiac disease, the variability inherent in these Doppler diastolic measurements must be examined. Therefore, the present investigation was designed to determine the sources of technical and biologic variability in Doppler echocardiographic indexes of left ventricular diastolic performance, to quantify their magnitude, and to compare their relative importance. Technical variability due to the reader was small for each of the six Doppler indexes, both for the comparisons of measurements obtained in the single subject and for analysis of the mean differences between groups of subjects. Variability due to the technician was larger than for the reader, and became sizeable when measurements obtained in a single subject were compared; however, variability due to the technician was negligible when mean differences between groups of subjects were analyzed, and none of these differences achieved statistical significance. Day-to-day variability (i.e., biologic variability) was larger than technical variability (reader and technician) for the great majority of the comparisons, both in the individual (28 of 30 comparisons) and group data analyses (27 of 30 comparisons). Four Doppler indexes assessing early diastolic events (i.e., isovolumic relaxation, duration of the early diastolic peak of flow-velocity, rate of decrease of flow-velocity in early diastole, and maximal early diastolic flow-velocity) did not show statistically significant changes due to day-to-day variability, when group data were analyzed. However, the two Doppler indexes assessing late diastolic events (i.e., maximal late diastolic [atrial] flow velocity, and the ratio between maximal early and late flow-velocity) did show significant change on a day-to-day basis ($p<0.05$).

In conclusion, Doppler indexes that represent a measure of the relaxation and early filling phases of diastole showed good reproducibility for group data analysis. Hence, these Doppler indexes can be utilized to assess left ventricular diastolic function noninvasively in large groups of patients, including the serial evaluation of the effects of treatment intervention. However, extensively utilized indexes assessing the atrial contribution to ventricular filling, such as maximal late (atrial) diastolic flow-velocity and the ratio between maximal early and the late diastolic flow-velocities, have a relatively large variability for both individual and group data analysis; therefore, conclusions based on these late diastolic indexes should be drawn with caution.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04146-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Smooth muscle growth and laminine rich matrix

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


| Name | Title | Affiliation |
|---|---|---|
| Zhou Yifu, M.D. | Special Volunteer | CB NHLBI |
| Edmund Yang, B.S. | Special Volunteer | CB NHLBI |
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |
| Edith Speir, B.S. | Biochemist | CB NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)


Recent studies by other groups have demonstrated that the extracellular matrix confers much more than a type of extracellular support or cement and in fact plays an important role in the control of cell growth, differentiation and migration. Attachment of cells to matrix and invasion of cells into matrix, (including malignant metastases) varies with the type of matrix. One type of matrix gel has been shown to promote a differentiated phenotype in a variety of cells. The gel is extracted from the abundant extracellular matrix of the EHS tumor. We hoped that this gel extract could prevent the differentiation of cultured smooth mucle cells which prevents their contractile properties from being studied in vitro. We found that passaged rat aortic smooth muscle cells attached much faster on the gel and on plastic and that in 10% fetal calf serum the cells on matrix grew faster when matrix was added to postconfluent serum starved cells. However, while the matrix supported these cells in the period of starvation, it also blunted the growth response to refeeding the cells with serum. By light microscopy the cells were seen to pick up before reaching confluence, but individually the cells were indistinguishable by electron microscopy from cells grown on plastic. Nor was a voltage dependent calcium channel induced by growing the cells on a gel, as indicated by the fura-2 signal in response to depolarization of potassium. Thusfar, we can only conclude that the gel extract exerts biphasic effects on growth as well as some phenotypic alterations, but does not confer a more differentiated type of electrophysiological response.





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

Project No. Z01 HL 04146-01 CB

Project Description:

Smooth muscle cell (SMC) proliferation - a feature of both atherosclerosis and hypertension - is known to be inhibited by heparin. SMC's, like other cells, may thus be influenced in a paracrine fashion by other glyocosaminoglycans and glycoproteins in the extracellular matrix (ECM). One such matrix - the gel extract of the laminin-rich ECM of the EHS tumor - promotes a differentiated phenotype in neuronal and germ cells. We thus tested the effects of this gel on passaged I$^{o}$ rat aortic SMC. Compared to cells grown in 10% fetal bovine serum (FBS) on plastic, subconfluent SMC's on EHS gel in 10% FCS grew twice as fast, as indicated by counting and 3HTdR incorporation ($p<.001$), even after correcting for the faster attachment of cells on the gel. The gel also enhanced growth when overlaid onto postconfluent serum-starved cells. Yet the response to refeeding with serum was blunted by the gel in dose-dependent fashion, by as much as 75%. Phenotypic alterations were noted by light and electron microscopy. We conclude that a laminin-rich extract of ECM exerts complex effects on SMC growth that are serum-dependent, but independent of attachment rate and seeding density.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04147-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Pharmacokinetics of endothelial cell growth factor

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


| | | |
|---|---|---|
| Todd K. Rosengart, M.D. | Medical Staff Fellow | SB NHLBI |
| John P. Kupferschmidt, M.D. | Medical Staff Fellow | SB NHLBI |
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |
| Ellis Unger, M.D. | Senior Staff Fellow | CB NHLBI |
| Thomas Maciag | American Red Cross, Bethesda, Md | |
| Richard E. Clark, M.D. | Chief, Surgery Branch | SB NHLBI |


COOPERATING UNITS (if any)

American Red Cross
Surgery Branch, NHLBI

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 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.)


Because we have previously found that a subcutaneus injection of basic fibroblast growth factor over 12 hours in the rat could cause endothelial cell DNA synthesis, we sought to learn something about the pharmacokinetics of these injections. Because of the limited availability of these growth factors and previous observations that heparin could enhance the binding of the acidic form of fibroblast growth factor, also known as endothelial cell growth factor, we selected this growth factor and injected it with and without heparin into rats intravenously, after pretreatment with sodium iodide to prevent binding of the radioactive iodine moiety which we used to label the endothelial cell growth factor. We found that the radiolabeled growth factor bound to all organs and in particular to the kidney, followed by the liver, on a dry weight basis. The least binding was to the brain. Because the kidney and liver have porous capillaries and filter the plasma through a large amount of subendothelial tissue rich in glycosaminoglycans, we suggest that the endothelial cell growth factor might bind to these components of the extracellular matrix. The low amount of binding to brain could relate to the very tight junctions between endothelial cells which constitute the blood-brain barrier. We also found that heparin enhanced the binding of ECGF to all organs, as it does to cultured cells. The exception was the kidney, which bound less ECGF when it was injected with an excess of heparin, perhaps indicating that the ECGF heparin binding sites were already occupied. The 4 fold increase in the circulating half life of the growth factor (to 60 seconds) and the enhanced endothelial binding (to organs other than kidney) suggest that heparin may enhance the utility of ECGF as an investigative therapy to promote angiogenesis and wound healing.




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

Project Description:

Endothelial cell growth factor (ECGF) is a potent mitogen for human endothelial cells which is potentiated by heparin *in vitro*. We have attempted to characterize the pharmacokinetics and distribution of ECGF following intravenous injection in the rat. ECGF purified from bovine brain was iodinated to a specific activity of approximately 2-4x$10^5$ cpm/ng protein. Eight rats (250 gms) were pretreated with sodium iodide followed by intravenous injection of 25 ng ECGF (0.125 ml) with or without heparin (2.5 U/ng protein). Serial blood samples were obtained, followed at 5 minutes by organ harvest, lyophilization and gamma counting. The $^{125}I$ remained protein bound as determined by precipitation with trichloroacetic acid. The half-life of circulating ECGF was 17 seconds without heparin and 60 seconds with heparin. Distribution of ECFG is as follows:

| Tissue | cpmx$10^3$/gm Dry Tissue (+/- SD) | Tissue | cpmx$10^3$/gm Dry Tissue (+/- SD) |
|---|---|---|---|
| Kidney | 1680.0±439.7 | Diaphragm | 33.3±21.6 |
| Liver | 1140.5±235.0 | Heart | 30.5±4.1 |
| Spleen | 432.7±300.5 | Aorta | 21.0±6.7 |
| Thyroid | 62.7±7.5 | Fat | 7.3±4.1 |
| Lung | 56.3±25.0 | Brain | 5.3±0.6 |

Heparin increased ECGF binding to all organs counted except kidney, which was decreased ($p<.01$). These data would suggest that ECGF can be delivered as a pharmacologic agent and therefore may be useful for modulation of angiogenesis *in vivo*.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04148-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Heparin treatment of ischemia: Attempt to promote angiogenesis

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

| | | |
|---|---|---|
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |
| Edith Speir, B.S. | Biochemist | CB NHLBI |
| Fernando Bazoberry, M.D. | Special Volunteer | CB NHLBI |
| Sidney Yoon, B.S. | Special Volunteer | CB NHLBI |
| Elizabeth Talbot | Special Volunteer | CB NHLBI |
| Edmund Yang, B.S. | Special Volunteer | CB NHLBI |
| Cedric Sheffield, M.D. | Staff Fellow | CB NHLBI |
| Ellis Unger, M.D. | Senior Staff Fellow | CB NHLBI |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB NHLBI |

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI- NIH, Bethesda, Md.

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3 | 2 | 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 this project, begun in September 1985, we have been trying to extend the findings that heparin and certain non-anticoagulant heparin fragments can promote the angiogenesis produced in the chick chorioallantoic membrane and in the rabbit cornea by tumor fragments or purified growth factors. That such an effect would occur with systemic administration is not at all certain because the heparin administration in the other bioassays was administered by direct extravascular administration so that the heparin was present along a concentration gradient in the extracellular space and was not first exposed to the plasma and the endothelial cells lining the vascular lumen. Heparin had previously been shown by other workers to promote endothelial cell migration if present in a gradient. We, and others, have shown that heparin binds to endothelium. In addition, heparin binds proteins present in the plasma Most importantly, however, it is still not established that myocardial ischemia results in angiogenesis as discussed in another annual report. As described in this abstract, we have found that in rats made increasingly ischemic-by graded doses of isoproterenol designed to produce ischemia without infarction-heparin and certain nonanticoagulant tetrasaccharide fragments produced a beneficial effect: a reduction in mortality and myocardial infarct size after left anterior descending coronary artery ligation 24 hours after cessation of the isoproterenol and the heparin (or saline). The usual effects of heparin such as its anticoagulant action appear not to account for this beneficial effect as indicated in the abstract. To confirm directly that this is an angiogenic effect we are counting capillary density and capillary/myocyte ratios in these hearts and quantifying endothelial DNS synthesis by tritiated thymidine autoradiography.

Project Description:

Heparin (H) and some nonanticoagulant H fragments, potentiate tumor angiogenesis but alone are not angiogenic. We postulated that cardiac angiogenesis induced by ischemia, might be enhanced by heparin. We developed a rat model in which ischemia was caused by isoproterenol (I) 20-80 ug/kg sc bid over 4 d. This dose produced lactate and EKG changes but did not cause subendocardial necrosis. We gave half the rats H 250 u/kg sc bid, or saline (S). Twenty-four hrs after the last dose, and 4 hrs after LAD ligation, H rats had a lower mortality than S rats (33.1 vs 43.3%, $p<.05$) and surviving H rats had smaller infarcts (35.7 vs 42.8%, $p<.001$) by tetrazolium. Risk regions were H:44$\pm$ 6.3%, S:51$\pm$8.1%, $p<.05$. Groups did not differ in PTT, HCT, wbc, plt count, bleeding T, k+, fibrinogen at thoracotomy, or in HR and BP response to I. Further, a nonanticoagulant H tetrasaccharide showed similar effects. Thus, heparin treatment prior to coronary ligation reduces risk region, and decreases mortality and infarct size at subsequent coronary ligation through a nonanticoagulant mechanism. These results are consistent with the hypothesis that heparin enhances ischemia-induced angiogenesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04149-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Heparin can enhance or inhibit smooth muscle cell growth

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


| | | |
|---|---|---|
| Zhou Yifu, M.D. | Special volunteer | CB NHLBI |
| Edmund Yang, B.S. | Special volunteer | CB NHLBI |
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |
| Edith Speir, B.S. | Biochemist | CB NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)


In recent years a great deal has been learned about the inhibition of smooth mucle cell growth by heparin, a phenomenon which may prove to be of major importance in the inhibition of atherosclerosis. The mechanism of this effect is unknown. In other studies in this laboratory heparin has been found to enhance the action of acidic fibroblast growth factor, which is known to cause smooth muscle proliferation in vitro. We have also observed the heparin alone at very low doses could at times stimulate the growth response of fibroblasts to serum. Consequently, we thought it possible that heparin in very low doses might actually enhance smooth muscle cell growth and this is what is reported in this accompanying abstract.




PHS 6040 (Rev. 1/84)

GPO 914-918

Project No. Z01 HL 04149-01 CB

Project Description:

We have found complex effects on smooth muscle cell (SMC) growth of a gel extract of the EHS tumor which is rich in extracellular matrix (ECM). The ECM components heparan and heparin are known to inhibit smooth muscle cell growth. In this study we found that after attachment, very low doses (.01-0.1 ug/ml) stimulated of heparin stimulated growth of subconfluent 6th passage rat aortic SMC's on plastic, in the presence of 10-15% fetal calf serum. Inhibition began only at 1 ug/ml. This concentration almost completely prevented a growth response to simultaneous addition of serum. In cells grown in the gel, 10 to 100 ug/ml heparin was required to inhibit the growth response to serum. We thus conclude that very low dose heparin can enhance smooth muscle cell growth in serum while higher doses inhibit SMC growth. The dose response was shifted to be right by an extract of ECM rich in laminin, collagen Type IV and heparin sulfate proteoglycan. We therefore conclude that heparin's effects on vascular smooth muscle cell growth are complex - varying with dose and presence or absence of matrix.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04150-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Lack of effect of heparin on cardiac hypertrophy

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


| | | |
|---|---|---|
| Cedric D. Sheffield, M.D. | Staff Fellow | CB NHLBI |
| Edith Speir, B.S. | Biochemist | CB NHLBI |
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .25 | .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.)


Heparin is known to inhibit smooth muscle growth in culture and in vivo following experimental carotid injury. Because in many cases cells increase in size before they divide, and because adult heart cells are capable only of an increase in size and incapable of cell division, we thought it possible that heparin might inhibit cardiac hypertrophy. Consequently in studies performed for other reasons, we were able to make incidental observations of the heart weight to body weight ratios of rats treated with either saline or isoproterenol. Using this rough estimate of hypertrophy we were able to confirm as others had shown that isoproterenol causes hypertrophy. However, heparin neither decreased the heart weight to body weight ratios of the saline controls or of the rats in whom hypertrophy had been created by isoproterenol infusions.




PHS 6040 (Rev. 1/84) GPO 914-916

Project Description:

Heparin and its larger degradation products have been reported to inhibit smooth muscle proliferation in culture and in vivo following experimental carotid balloon injury. Heparin also suppresses the mitogenic response of cultured fibroblasts to platelet derived growth factor. This suggests that heparin may act as a modulator of growth of other mesoderm-derived cells.

We tested the hypothesis that heparin inhibits the hypertrophy of nondividing cardiac myocytes in vivo. We administered normal saline or heparin (250 IU/kg sc bid) to 200-225 gram male Sprague Dawley rats given either saline or non-infarcting increasing doses of isoproterenol (20-80 ug/kg sc bid) for 4 days. On day 5 wet heart weight to body weight ratios were determined. The ratios are expressed as the mean x 1000 $\pm$ the standard deviation for 10 determinations in each of 4 groups.

| | Saline | Isoproterenol |
|---|---|---|
| Saline | 4.32 $\pm$ .58 | 5.59 $\pm$ .54 |
| Heparin | 4.56 $\pm$ .43 | 5.36 $\pm$ .78 |

The ratios for the isoproterenol/saline and isoproterenol/heparin groups are significantly greater than control by pooled t-test. There is no significant difference between the isoproterenol/saline and isoproterenol/heparin groups. In conclusion, these data show that heparin has no significant effect on the magnitude of isoproterenol-induced cardiac hypertrophy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04151-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Purification of myocardial heparin-binding growth factors

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

| | | |
|---|---|---|
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |
| Edith Speir, B.S. | Biochemist | CB NHLBI |
| Duc Nguyen | Special Volunteer | CB NHLBI |
| Edmund Yang, B.S. | Special Volunteer | CB NHLBI |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)

Basic and acidic fibroblast growth factors have recently being sequenced and cloned. They are known to be heat labile polypeptides of molecular weight 14,000-18,000. Their mitogenic activity has recently been demonstrated not just for fibroblast but now for glial cell, endothelial cells, smooth muscle cells, chrondrocytes and a few other mesenchymal cells. Their origins are now known not to be restricted just to the brain and to tumors and a large variety of cells, but not all, synthesize these factors in culture. In bioassays they are shown to cause angiogenesis and a role in embryogenesis and differentiation has been reported. Although the heart is a mesenchymal organ, it was not clear to us what role FGF might have in the normal, adult, highly differentiated heart. However, we were able to purify either FGF or peptide of similar molecular weight and similar mitogenic and inmunologic characteristics from the normal adult human left ventricle using the now standard techniques of heparin sepharose chromatography. We are now trying to find out if the protein levels of FGF are altered in ischemia and infarction.



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

Project Description:

Heparin-affinity chromatography of human left atrial tissue obtained at cardiac surgery and subjected to 6th or ex-vivo infarction yielded mitogenic activity for endothelial cells and immunoreactivity by slot-blot with antisera for acidid and basic fibroblast growth factors (FCF). We extended these findings using 58mg of histologically normal postmortem 70 year old male left ventricle. The tissue was homogenized and sonicated in IM NaCl, 0.01M Tris-EDTA pH7 .4, spun at 10,000g for 30', acidified to pH4.5 for 30', re-spun and the supernatant eluted fromheparin-sepharose with a NaCl gradient SDS-PAGE rvealed a single band of Mr14,000 from 1 to 1.6 M. This protein had intense mitogenic activity as measured by tritiated thymidine incorporation in Balb/c 3T3 fibroblasts. It reacted by slot-blot with highly specific polyclonal antisera raised against residues 1-12 and 33-43 of basic FGF and 7-16 of acidid FGF, suggesting that the band may be comigrating acidic and basic FGF-like peptides, whose lower Mr may reflect N- and C-terminal cleavage or a smaller translational product characteristic of human left ventricular myocardiaum. Although grossly and histologically normal postmortem findings do not guarantee biochemical normality, it seems unlikely that these proteins were synthesized as an agonal event since we have also found them in perfused quick-frozen dog and rat hearts extracted at $4^{\circ}C$. Bioassays and in vitro studies have identified a role for the FGF's in angiogenesis and other types of growth and differentiation. However, their role in the adult, differentiated, non-diseased heart is obscure.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04152-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Effects of vasoactive drugs on fibroblast proliferation

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


| | | |
|---|---|---|
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |
| Edith Speir, B.S. | Biochemist | CB NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)


Our interest in the possible effects of vasoactive drugs on fibroblast and endothelial cell proliferation derives from 3 lines of research. The first is biochemical evidence that systolic ionized calcium increases quickly in response to a variety of polypeptide growth factors just as it does in response to vasoconstricting agents such as epinephrine vasopressin or angiotensin. Some polypeptide growth factors were reported to have vasoconstrictive activity while by the same token some of the classical vasoactive agents which are smaller peptides or aminoacid derivatives have been found to promote cell proliferation or hypertrophy. In addition, there are a number of conflicting reports suggesting that some vasodilating agents may over the long run lead to increased vascularity in the heart. Since such agents tend to lower intracellular calcium, an indirect action to cause endothelial cell growth (such as the effect of stretching, flattening, increased wall tension or increased turbulence or flow across these cells), might account for the cell growth. Thus, for a variety of reasons, it is of interest to see whether vasoconstricting or vasodilating drugs have any direct effect on the proliferation of cultured fibroblast and endothelial cells. We began with the study of 3T3 fibroblasts and, as reported in this abstract, found only modest growth enhancement of the vasoconstrictive substances in the physiological dose range. The vasodilating drugs neither promoted growth by themselves nor blocked the growth effect of serum. These results are being followed-up using these and other drugs on endothelial cells. If the results are similar, this would certainly indicate that the in vivo situation is much more complex and cannot be modelled by isolated cultures of pure cell populations.

Project No. Z01 HL 04152-01 CB

Project Description:

Cytosolic Ca+2 rises quickly in response to platelet-derived, epidermal and fibroblast growth factors. That these were recently found to be vasoconstrictors while vasoconstricting alpha-adrenergic agonists promote hypertrophy of cultured cardiac myocytes, led us to test other vasoactive (and presumably Ca+2 modulating) drugs for growth effects. Tritated thymidine incorporation in confluent Swiss 3T3 fibroblasts in 7d-old 10% calf serum and DMEM indicated 3-5-fold increasis (vs. 30 fold for serum) for epinephrine (10E-6 to 1-E-4M), $CaCl_2$ (5X10E-4M), phenylephrine (10E-4M) and no effect of prazosin (10E-7 to 10E-5M), verapamil (10E-8 to 10E-6M), isoproterenol (10E-7M) or atrial natriuretic peptide (pro ANP103-126), 10-10E3 pg/ml). The growth effect of fresh serum was enhanced only by low doses epinephrine (10E-6M) or $CaCl_2$. unaffected by V or ANP, and reduced 25% by high doses of phenylephrine isoproterenol prazosin or prazosin plus propranolol. Thus while ephinephrine phenylephrine and $CaCl_2$ modestly enhance fibroblast growth, and so could affect the fibroblast respond in healing or myocardial hypertrophy, a variety of vasodilators had no effect. Other mesenchymal cells (endothelial, smooth and cardiac muscle) and other vasoactive drugs merit testing for similar effects, which could have relevance for such processes as angiogenesis, arteriosclerosis, and myocardial hypertrophy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04153-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Isolation of a canine myocardial fibroblast mitogen

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

| | | |
|---|---|---|
| Benjamin Calvo, M.D. | Special Volunteer | George Washington University |
| Michael Klagsbrun, Ph.D. | Assoc. Professor | Harvard Medical School |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB NHLBI |
| Edith Speir, B.S. | Biochemist | CB NHLBI |
| Joachim Sasse, Ph.D. | Post-doctoral Fellow | Harvard University |
| Judah Folkman, M.D. | Prof. of Surgery | Harvard Medical School |
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |

COOPERATING UNITS *(if any)*

George Washington University, Washington, DC
Department of Surgery, Children's Hospital, Boston, MA
Harvard Medical School, Boston, MA

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)*

This study demonstrated the presence in infarcted dog myocardium of a number of polypeptides with affinity for heparin sepharose, the larger of which immunoreacted was a polyclonal antiserum to the aminoterminus of basic fibrobast growth factor. The smaller bands on the SDS gel, approximately 14,000 molecular weight, were mitogenic but not immunoreactive, possibly suggesting that they represent aminoterminus cleavage of fibroblast growth factor which is consistent with recent reports of protease cleavage. Such cleavage is enhanced in acidic conditions such as are found in the infarcted myocardium. That only limited cleavage, without loss of mitogenic capacity, occurs in myocardial infarction, may be important in understanding the angiogenic and wound healing responses in myocardial infarction.



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

Project No. Z01 HL 04153-01 CB

Project Description:

Heparin-affinity chromatography has allowed the purification from brain and tumors of a family of polypeptides of MW 15-18,000 that are angiogenic *in vivo*, chemotactic for capillary endothelial cells *in vitro* and mitogenic for capillary endothelial cells, fribroblasts, and smooth muscle cells *in vitro*. To determine whether such factors were present in the ischemic heart, we excised the infarct zones of foxhounds 3 hours after left anterior descending coronary artery ligation. After homogenization and centrifugation, the supernatant was acidified to pH 4.5, and the proteins precipitated with 50%, then 95% $(NH_4)_2SO_4$. The resuspended pellet was dialyzed against Tris/EDTA, run twice through a heparin-sepharose column and eluted with NaCl gradients. In confluent starved Balb/c 3T3 cells fractions eluting from 1 to 2 M produced a growth phenotype and increased incorporation of tritiated thymidine. SDS-PA gel electrophoresis showed several bands, from 14-18,000. These fractions immunoreacted by slot-blot with a polyclonal antiserum against the N-terminus of cationic fibroblast growth factor (FGF). A third heparin column yielded a single band at 14,000 which was mitogenic but not immunoreactive. Thus, infarcted myocardium yields several polypeptide mesenchymal mitogens, one of which is similar if not identical to bovine FGF, while the purified mitogen is not. These polypeptides may influence angiogenesis and wound healing.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04154-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Increase in FGF by RIA in myocardial infarction

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


| | | |
|---|---|---|
| Edmund Yang, B.S. | Special Volunteer | CB NHLBI |
| Pamela E. Karasik, M.D. | Medical Staff Fellow | CB NHLBI |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB NHLBI |
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)


Although the in vivo properties of basic fibroblast growth factor is unknown, and nothing is known about its regulation at the transcriptional or translational levels or about its release, the in vitro mitogenecity of FGF for fibroblasts, smooth muscle cells and especially for endothelial cells, raises the possibility that it is important in wound healing and angiogenesis. To produce myocardial necrosis without contamination by blood borne elements such as platelets or white cells which might contain growth factors, rats hearts were exposed to 60 minutes of ex vivo incubation in saline at 24°. To assay this tissue it was necessary to develop a sensitive and specific RIA for basic FGF using polyclonal antisera raised against synthetic peptides representing aminoacids 1 to 24 and 33 to 43 of basic FGF. A wide variety of extraction conditions and RIA buffers were used to maximize sensitivity. Specificity was partially confirmed by parallel curves using serial dilutions of homogenized heart and additions of recombinant basic FGF to RIA buffer and to homogenized heart. Further proof of the specificity in this assay is being sought using western blotting and non denaturing techniques such as gel filtration and immunoprecipitation. With this radioimmunoassay the normal rat heart was found to contain approximately 9 mg per gram while the infarcted hearts contained twice that amount. The mitogenecity of the necrotic myocardium was only mildly enhanced compared to that of controls, suggesting that the FGF immunoreactivity may not be completely indicative of enhanced bioavailability. Alternatively myocardial necrosis may result in the release of growth inhibitors such as acidosis, hyperkalemia or specific polypeptides.




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

Project Description:

The angiogenic growth factors, acidic and basic fibroblast growth factor (aFGF and bFGF), are present in normal myocardium. We hypothesize that myocardial ischemia and/or infarction stimulates production or release of bFGF which, in turn, stimulates coronary collateral formation. As a model of myocardial ischemia progressing to infarction without inflammation we exposed 8 rat hearts to 60 minutes of *ex vivo* incubation in saline at 24°C. Hearts were extracted in the presence of protease inhibitors by homogenization in 3 ml saline, sonication, and centrifugation for 30 minutes at 30,000 X g. Radioimmunoassay, using a polyclonal antisera against bFGF amino acids 1 to 24 and sensitive to 40 pg, revealed bFGF levels rose from 8.85 $\pm$ 0.38 pg/mg in controls to 17.40 $\pm$ 2.53 pg/mg in *ex vivo* injured hearts ($p<.001$). Mitogenicity of *ex vivo* injured heart extracts on Balb/C 3T3 fibroblasts was also significantly higher than in controls. We conclude that bFGF is made more available with myocardial injury either by de novo synthesis or by release of bFGF from cellular or matrix bound stores. Regardless of the mechanism, the increased bioavailability of bFGF with myocardial injury may play a role in coronary angiogenesis or the healing of infarction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04155-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Myocardial acidosis releases basic fibroblast growth factor

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


| | | |
|---|---|---|
| Edmund Yang, B.S. | Special Volunteer | CB NHLBI |
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB NHLBI |


COOPERATING UNITS (*if any*)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.*)


Having shown an increase in basic FGF immunoreactivity of the myocardium that becomes necrotic over the hour following excision of the heart, we sought to determine what aspects of the ischemic and/or necrotic process accounted for this increased immunoreactivity. Because it seemed unlikely that basic FGF could be synthesized in such a short time and because we and others have shown that basic FGF is present in extracellular matrix and that FGF can be extracted with acid, we hypothesized that the acidosis of ischemia might release basic FGF from extracellular matrix and prevent the binding of intracellular stores of FGF to matrix on lysis of these cells. We found that acidosis to the extreme limits of that found in necrotic tissue was able to reproduce the effects of the ischemia itself which we take to be partial evidence in support of the hypothesis.




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

Project No. Z01 HL 04155-01 CB

Project Description:

Using *ex vivo* incubation of rat myocardium in normal saline as a model of myocardial ischemia, we showed that basic fibroblast growth factor (bFGF) is released from myocardium following 60 minutes of incubation. Although bFGF could be synthesized and released by ischemic myocardial cells, we hypothesized that pre-existing stores of bFGF are released from the extracellular matrix and made more bioavailable by myocardial acidosis. We therefore extracted control and 60 minute *ex vivo* injured hearts in pH extraction buffers ranging from 7.5 to 4.5 to determine whether decreasing pH releases bFGF and, if so, what pH simulates the ischemia mediated bFGF release. With decreasing pH, myocardial bFGF levels in control hearts rose significantly ($p<.01$), toward the levels of the ischemia injured hearts. Below pH 6.5 bFGF levels in the two groups paralleled each other. We conclude that myocardial acidosis can simulate ischemia/infarction stimulated release of bFGF. Given that pH 6.5 is the lower limit of tissue pH *in vivo* during myocardial ischemia, it is probable that ischemia induced acidosis causes release of bFGF from extracellular matrix, which then may play a role in stimulating coronary collateral growth.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04156-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

In vivo cardiac endothelial mitogenicity of fibroblast growth factor

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


| Name | Title | Affiliation |
|---|---|---|
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |
| Edith Speir, B.S. | Biochemist | CB NHLBI |
| Michael Klagsbrun, Ph.D. | Assoc.Professor | Harvard Medical School |
| Fernando Bazoberry, M.D. | Special Volunteer | CB NHLBI |
| Elizabeth Talbot | Special Volunteer | CB NHLBI |
| Judy Hung | Special Volunteer | CB NHLBI |
| Victor Ferrans, M.D. | Chief,Section on Ultrastructure | PB NHLBI |


COOPERATING UNITS (if any)

Section on Ultrastructure, Pathology Branch, NHLBI
Department of Surgery, Children's Hospital Medical Center
Harvard Medical School

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .5 | .25 | .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.)


The actions, if any, of the fibroblast endothelial growth factors in vivo are unknown. Naturally because they are extracted from intact organs, and especially from cells and culture, some function is likely. In cultured cells the endothelial and fibroblast growth factor family causes proliferation of fibroblast endothelial cells and smooth muscle cells and a variety of other mesenchymal cells. Because no single growth factor has been able to cause cell division, once cultured endothelial cells become confluent, one might not expect that these growth factors would have an effect in vivo, where the endothelial cell turnover rate is even slower than in the confluent cultured cells. Nevertheless, we found that in normal rats injections of large amounts of fibroblast growth factor caused endothelial cells to undergo DNA synthesis. Obviously, this may prove to be useful to enhance angiogenesis or wound healing.

Project Description:

The basic fibroblast growth factors (FGF) are mitogenic for fibroblasts, endothelial cells and smooth muscle cells in vitro. Angiogenesis has been demonstrated in vivo by direct (extralumenal) high dose, slow-release application to the chick chorioallantoic membrane or the avascular rabbit cornea. Since FGF is no longer mitogenic once endothelial cells are confluent, cannot exceed maximal serum mitogenicity, and has a short half-life, an in vivo effect at a distance from a subcutaneous (SC) dose in the normal adult animal would not be expected. Yet we found that, in the 250 g male rat, 750 ng of SK-1 hepatoma-derived growth factor (purified by cation exchange and heparin-Sepharose affinity chromatography injected in 5 doses of 5 ml saline SC,q2h, followed by $^{3}$H-thymidine 12 and 36 h later, resulted in a 7-fold increase in DNA-synthesizing cells, primarily in capillaries and venules. Thus FGF-like peptides may have, in addition to their autocrine or paracrine effects, an endocrine role.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04157-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Endothelial cell growth factor binds to endothelium in vivo

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


| | | |
|---|---|---|
| Todd K. Rosengart, M.D. | Medical Staff Fellow | SB NHLBI |
| Victor J. Ferrans, M.D. | Chief, Sec. on Ultrastructure | PB NHLBI |
| Ward Casscells, M.D. | Senior Staff Fellow | CB NHLBI |
| John P. Kupferschmidt, M.D. | Medical Staff Fellow | SB NHLBI |
| Thomas Maciag, Ph.D. | American Red Cross, Bethesda, Md | |
| Richard E. Clark, M.D. | Chief, Surgery Branch | SB NHLBI |


COOPERATING UNITS *(if any)*

American Red Cross
Surgery Branch, NHLBI

LAB/BRANCH

Cardiology Uranch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)*


Because the failure of superficial endothelial injuries to heal appears to play a role in the development of atherosclerosis and vasospasm, factors which could speed the healing are of interest for these processes as well as for the potential to enhance angiogenesis and thus prevent myocardial infarction. In this study, radioiodine labelled endothelial cell growth factor was prepared using endothelial cell growth factor (purified from bovine brain by heparin sepharose chromatography) and lactoperoxidase. The radiolabelled growth factor was shown to be active as an endothelial cell mitogen. In anesthetized rats, one carotid was denuded using an embolectomy balloon and the denudation was confirmed by Evans blue staining as well as by scanning electron microscopy. The animals were then injected intravenously with the radiolabel growth factor with or without heparin. Heparin was found to increase the binding to the intact carotid. The denude carotid bound 8 times as much ECGF as normal caroid, presumably reflecting binding to the extracellular matrix. This was supported by the fact that premixing of the growth factor with heparin decreased the binding to the matrix, although it did not return it to normal. These were the first studies of the binding of endothelial cell growth factor in vivo and they lend support to the idea that such growth factors may be useful to promote endothelial healing, which might be helpful for wound healing, seeding of vascular prosthesis, and to promote angiogenesis.





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

Project Description:

Endothelial cell growth factor (ECGF) in the presence of heparin produces a significant increase in endothelial cell (EC) growth *in vitro* and may prove useful for endothelial regeneration and endothelial seeding *in vivo*. We have characterized the intravascular binding of ECGF with and without heparin to intact and balloon-denuded rat carotid artery segments. These data were compared with *in vitro* binding properties.

ECGF purified from bovine brain was iodinated to a specific activity of approximately 1-2 X $10^5$ cpm/ng protein and shown to have retained biological activity as determined by *in vitro* mitogenic assay. *In vitro* binding studies measured $^{125}$I-ECGF incorporation by confluent human umbilical vein endothelial cells (HUVEC) grown in standard media with ECGF and heparin. Carotid artery segments were denuded of endothelium in 350 gm rats (n=14) by passing a saline-filled 2F embolectomy catheter three times across the left carotid. Complete denuding of the carotid artery was verified by Evans blue staining and by scanning electron microscopy. Five minutes after intravenous injection of 40 ng (0.2 ml) of $^{125}$I-ECGF with or without heparin (2.5 U/ng ECGF), and at steady-state ECGF serum concentrations, the animals were perfusion-fixed with ice-cold glutaraldehyde/paraformaldehyde solution and both carotid arteries were excised. ECGF binding as determined by gamma counting, was as follows:

| Treatment | Right Carotid-Intact (cpm ± S.D.) | | Left Carotid-Denuded (cpm ± S.D.) | |
|---|---|---|---|---|
| ECGF | 347 ± 249 | p=0.007 | 2658 ± 777 | p=0.02 |
| ECGF/Heparin | 978 ± 388 | | 1591 ± 557 | |

Saturation of binding occurred at an ECGF concentration of 10 ng/ml (200 ng total dose) as determined by injection of varying amounts of ECGF with heparin in different rats. *In vitro* studies were similar in that: i) saturation occurred at an ECGF concentration of 10 ng/ml, ii) heparin enhanced ECGF binding to endothelial cells as with the right carotid (intact) but, iii) heparin decreased non-specific binding of ECGF similar to results with the left carotid (denuded). Finally, maximal ECGF binding obtained with heparin in rats was similar to maximal *in vitro* ECGF binding (0.07 ng/$cm^2$ vs 0.024 ng/$cm^2$, respectively). ECGF produced maximal *in vitro* EC growth at these concentrations. These studies suggest that use of IV ECGF can accelerate regeneration of denuded autologous surfaces and may be efficacious for rapid coverage of synthetic vascular grafts. Studies using continuous infusion of ECGF to regenerate denuded endothelium are currently in progress.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04158-01 CB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Catalytic thermal tip catheter for angioplasty

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

| | | |
|---|---|---|
| David Y. Lu, M.D. | Staff Associate | CB NHLBI |
| Robert L. Bowman, M.D. | Chief, Lab. of Technical Development | TD NHLBI |
| Martin B. Leon, M.D. | Senior Investigator | CB NHLBI |

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .6 | .6 | 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.)


Thermal angioplasty is a newly developed technique that employs a heated metallic tip to thermally ablate atherosclerotic plaques for recanalization of obstructed peripheral arterial blood vessels. However, the current Laser Thermal Probe employs a high power continuous argon laser which is large in size and high in cost. Last year, we developed an electrically heated thermal tip catheter as a possible alternative to the Laser Probe, and preliminary testing of this device in an in vivo animal model appears promising.

Another economical method of heating a metallic tip is to harvest the chemical energy from the combustion of hydrogen gas. This energy can be released and harvested inside a catheter tip in a safe manner by utilizing a palladium sponge catalyst which initiates and maintains the chemical combustion in a controlled fashion. A prototype catalytic thermal tip catheter has been designed and fabricated for in vitro testing. A temperature feedback control device has been added to avoid excessive tissue heating with the aim of minimizing incidences of vessel wall perforation. Extensive in vivo testing in an atherosclerotic animal model is planned prior to consideration of clinical human trials.




PHS 6040 (Rev. 1/84)

GPO 914-918

Project No. Z01 HL 04158-01 CB

Project Description:

Laser thermal angioplasty (TA) using metal-tipped quartz fibers has been used successfully to recanalize obstructed human peripheral arteries and preliminary studies are ongoing in patients with coronary artery disease. A new catalytic thermal tip (CTT) catheter has been designed providing an alternative energy source for TA without the expense and technical support needed for a laser operated device. The CTT utilizes the principle of combustion energy from a stoichiometric ratio of oxygen and hydrogen gases catalyzed by a small piece of palladium sponge within the tip. Gas flow regulates CTT temperature (T) which is monitored by a copper-constant thermocouple inside the metal tip. A 3F prototype CTT was studied in air and saline, alone and with human atherosclerotic aortic segments. Heating was faster in air (>350°C in <1sec) than saline (T (max) of 170°C in 5 sec, t 1/2=0.6 sec), but thermal relaxation was faster in saline (t 1/2=1.5 sec) than air (t 1/2=8 sec) due to rapid heat convection in saline. In both air and saline, CTT-tissue contact effects were directly related to T at the tip; histologic thermal injury began at T>180°C but ablation with crater formation, charring, and polymorphous vacuoles did not occur until T>325°C. Effective tissue ablation in saline required initial vaporization of the saline at the CTT-tissue interface.

We conclude that the CTT catheter is safe, inexpensive, and results in efficacious tissue ablation which can be regulated by T feedback monitoring and may be preferable alternative to laser TA.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04159-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Negative left ventricular pressure (diastolic suction) in human subjects

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


| | | |
|---|---|---|
| James E. Udelson, M.D. | Medical Staff Fellow | CB NHLBI |
| Stephen L. Bacharach, Ph.D. | Imaging Physicist | NM NHLBI |
| Richard O. Cannon, M.D. | Senior Investigator | CB NHLBI |
| Robert O. Bonow, M.D. | Chief, Nuclear Cardiology Sec. | CB NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Nuclear Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)


While it is generally accepted that left ventricular relaxation is an active, energy requiring process, controversy exists as to whether the left ventricle is capable of developing a "suction" pressure that would enable filling to take place even with no or low source pressure to drive filling. Previous studies have demonstrated negative left ventricular pressures in non-physiologic animal models (i.e., with mitral valve occlusion) and in humans with mitral stenosis. It has been speculated that negative LV pressure or "suction" might be present and beneficial in normal humans with exercise. In the present study, we used isoproterenol infusion to simulate conditions of exercise (during catheterization study of coronary reserve in patients with chest pain and normal coronary arteries), and found that negative LV pressure could in fact be demonstrated in almost all cases. The results shed light on the mechanisms by which the normal human heart is able to dramatically increase cardiac output during exercise at high heart rates, when left ventricular filling must be extremely rapid.




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

Project Description:

Negative left ventricular (LV) pressure (P) (diastolic (D) "suction") is seen in animals at low LV volume (V) and afterload, and in humans with mitral stenosis. To assess whether this may occur in normal humans, we studied 8 pts with chest pain and normal coronary arteries with simultaneous gated measurement of LV V (radionuclide angiography) and P (micromanometer catheter); two minutes of data were obtained in the basal state (B), during Isoproterenol (I), and during atrial pacing (PAC) to similar heart rates. Compared to PAC, I reduced end systolic (ES) V ($p<.005$), ESP ($p<.005$), and the half time of P decline after peak-dp/dt (T 1/2, $p<.01$). Negative diastolic P developed in 7/8 pts during I, (range -.5 to -2.4 mmHg, mean -1.1), but in only 1/8 during PAC (-.5 mmHg). During I, the minimum LVP was related to ESP ($r=.82$), and from B to I, the reduction of minimum LVP was related to the % change in ESV ($r=.85$), ESV/EDV ratio ($r=.92$), and the change in T 1/2 ($r=.9$). Hence, negative LVP may be seen during I, a situation similar to exercise, and is related to LV systolic unloading and deformity, and rapid isovolumic re-relaxation. This negativity contributes to the LV-left atrial pressure gradient, enhancing the rapid rate of LV filling required to maintain high cardiac output at elevated heart rates.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04160-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Verapamil improves silent perfusion abnormalities in hypertrophic cardiomyopathy

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



| | | |
|---|---|---|
| James E. Udelson, M.D. | Medical Staff Fellow | CB NHLBI |
| Stephen L. Bacharach, Ph.D. | Imaging Physicist | NM CC |
| Arthur Van Lingen, Ph.D. | Fogarty Fellow | NM CC |
| Stephen M. Larson, M.D. | Chief, Dept. Nuclear Med. | NM CC |
| Robert O. Bonow, M.D. | Chief, Nuclear Cardiology | CB NHLBI |



COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Nuclear Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .3 | .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.)


Previous studies from our laboratory demonstrated that over one-half of asymptomatic patients with hypertrophic cardiomyopathy have silent, stress-induced perfusion defects on thallium-201 tomographic scanning. Repetitive episodes of myocardial ischemia may eventuate in myocardial damage and later left ventricular dysfunction, or provide a setting for genesis of malignant dysrhythmias. In the present investigation, we studied the efficacy of the calcium channel blocker verapamil in preventing these stress-induced perfusion abnormalities. We found that the majority of patients with perfusion defects off medication had improved regional myocardial perfusion with verapamil, and in many cases perfusion became completely normal. These data have important therapeutic implications for this group of patients, and long-term follow-up will attempt to define the prognostic aspects of the finding of silent ischemia.





PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

Recent studies have shown that stress induced reversible thallium (TL) perfusion defects (PDs) are common in asymptomatic or mildly symptomatic patients with hypertrophic cardiomyopathy (HCM). To assess the effect of therapy, we studied 22 asymptomatic HCM patients, age 18-52 (mean 28.5), with stress TL tomography before and again during verapamil (V), mean dose 453 mg/day. Treadmill time was slightly greater during V (21.2 vs 22.0 min $p<.05$), but double product was similar (26.9 vs 25.8 X $10^3$, p=NS). Two midventricular short axis stress images per study were divided into 5 regions each and analyzed blindly by 3 observers on a 0-2 scale, with scores averaged (score $\leq 1.5$ = defect, change $\geq .5$ = significant change). On no drugs, 13 patients had reversible PDs, averaging 3.5 regions per patient. During V, perfusion scores of 9 of these 13 patients (69%) improved, with overall improvement in 31 of 45 (69%) initial PDs. In 6 patients with regional PDs (average 4 patients), V normalized all regions. No region worsened during V. Thus, in the majority of these asymptomatic patients, regional myocardial PDs improved during V, in some cases completely. These data suggest that V may reduce stress induced episodes of silent ischemia in asymptomatic patients with HCM.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04161-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Peak filling rate by radionuclide angiography: Effect of Normalization Parameter

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


| | | |
|---|---|---|
| James E. Udelson, M.D. | Medical Staff Fellow | CB NHLBI |
| Robert O. Bonow, M.D. | Chief, Nuclear Cardiology Sec. | CB NHLBI |
| Stephen L. Bacharach, Ph.D. | Imaging Physicist | NM NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Nuclear Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .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.)


Normalized peak filling rate (PFR), as derived from the radionuclide angiographic time-activity curve, is widely utilized in the investigation of diastolic performance of the intact heart. Because the nuclear technique reveals only relative and not absolute volume changes, the raw value of PFR, derived in counts per second, must be "normalized", or expressed in terms of a volume parameter obtained from the time activity curve. Most commonly, this is expressed as end-diastolic volumes per second. However, this makes the determination of PFR dependent on both end-diastolic volume as well as ejection fraction. The purpose of our investigation was to study the impact of alternative normalization parameters on results of previous studies which had used PFR normalized by end-diastolic volume. For intergroup comparisons between normal subjects and patients with hypertrophic cardiomyopathy, alternative normalization parameters importantly affected the group comparisons, owing to the large differences in the parameters between the groups (i.e. end-diastolic or end-systolic volume). For serial comparison of patients before and after an intervention, the direction, magnitude and statistical significance of the changes in PFR were almost always maintained with different normalization parameters. Our results indicate the importance of evaluating changes in the normalization parameter itself between groups or during an intervention, before changes in the normalized PFR may be ascribed to changes in diastolic performance.




PHS 6040 (Rev. 1/84)

GPO 914-918

Project No. Z01 HL 04161-01 CB

Project Description:

Peak filling rate (PFR) obtained by radionuclide angiography is usually normalized to end-diastolic volume (EDV). Since apparent changes in PFR after an intervention may reflect changes in EDV rather than changes in LV filling per se, alternative normalization methods may yield significantly different results. To assess the effect of normalization method, we studied 2 groups of patients at rest with interventions previously shown to enhance PFR normalized to EDV: 42 patients with hypertrophic cardiomyopathy (HCM) received verapamil, and 25 patients with single-vessel coronary disease (CAD) undergoing angioplasty (PTCA). In addition to EDV normalization, PFR was normalized by stroke volume (SV), end-systolic volume (ESV) and instantaneous volume at time of PFR (VPFR). Neigher interventin altered mean ejection fraction (EF); heart rate decreased in HCM after verapamil, but was unchanged by PTCA.

| | HCM | | CAD | |
|---|---|---|---|---|
| | control | verapamil | Pre PTCA | Post PTCA |
| PFR EDV/s | 3.2± 1.1 | 4.1±1.2* | 2.3±0.6 | 2.7±0.6* |
| SV/s | 4.3± 1.3 | 5.6±1.5* | 4.2±1.1 | 5.0±1.0* |
| ESV/s | 19.5±26.5 | 21.3±18.3+ | 5.1±1.7 | 6.3±2.2* |
| VPFR/s | 6.0± 2.1 | 7.3± 2.4* | 3.4±0.9 | 4.1±1.1* |

Values= mean ± SD *p<.001 + p=NS

All methods produced similarly significant results, except the ESV method in HCM patients. This reflected the finding that in some HCM patients with very high EF, a very small ESV underwent a relatively large (though quantitatively small) increase after verapamil. Although quantitatively different values may result, we conclude that in general, significant directional changes in PFR after an intervention are maintained regardless of the normalization parameter.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04162-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Influence of gender on SPECT thallium uptake and washout in normal subjects

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

| | | |
|---|---|---|
| James E. Udelson, M.D. | Medical Staff Fellow | CB NHLBI |
| Arthur Van Lingen, Ph.D. | Fogarty Fellow | NM CC |
| Patrick T. O'Gara, M.D. | Guest Researcher | CB NHLBI |
| Stephen L. Bacharach, Ph.D. | Imaging Physicist | NM CC |
| Lauren Chang | Guest Researcher | CB NHLBI |
| Stephen M. Larson, M.D. | Chief, Dept. Nuclear Med. | NM CC |
| Robert O. Bonow, M.D. | Chied, Nuclear Cardiology Section | CB NHLBI |

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Nuclear Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| .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.)

As quantitative analysis of Thallium-201 uptake and washout are increasingly used for the non-invasive detection of coronary artery disease, optimal definition of "normal limits" becomes imperative to maximize the sensitivity, specificity and predictive accuracy of this test. We and others have now pointed out that gender differences are present in thallium washout, suggesting that gender-matched normal data ranges may be important. In the next phase of this study, we will examine the physiology of this phenomenon in normal subjects, with serial analysis of whole body, blood, and serum thallium kinetics after exercise, as well as analysis of renal clearance and muscle mass. We will test the hypothesis that the relatively greater muscle mass in men acts as a "sink" for Thallium-201, affecting the analysis of washout from the myocardium.



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

Project No. Z01 HL 04162-01 CB

Project Description:

Optimal separation of disease states from normalcy requires precise knowledge of the range of normal data. Although relative regional activity of TL-201 differs between males (M) and females (F), the influence of gender on washout analysis (a parameter unaffected by regional attenuation fractors) in tomographic studies is not known. To investigate this, we studied 51 volunteers, 27 M and 24 F, ages 18-57, who underwent SPECT TL-201 scanning after treadmill exercise and at 3 hr rest. Circumferential profiles of initial relative uptake and 3 hr washout were determined for global and regional analysis. Peak rate-pressure product was greater in M than F ($p<.01$), peak HR was similar (183). Mean ratios of initial uptake were:

| | Ant/Sept | Ant/Inf | Sept/Lat |
|---|---|---|---|
| M | 1.03 | 1.16 | .84 |
| F | .95 | 0.98 | .86 |
| p value | <.001 | <.001 | N.S. |

| Mean 3 hr washout %: | Global | Ant | Sept | Inf | Lat |
|---|---|---|---|---|---|
| M | 45 | 46 | 44 | 43 | 47 |
| F | 50 | 50 | 49 | 49 | 52 |
| p value | <.02 | NS | <.02 | <.02 | <.01 |

Initial uptake differences were consistent with breat attenuation. However, washout was consistently higher in F than M, despite higher double product in M. This may reflect greater skeletal muscle TL-201 and therefore slower blood pool clearance in M. Hence, significant differences occur in both uptake and washout between M and F in TL-SPECT studies, providing further evidence that gender matched normal data bases are required for optimal indentification of patients with perfusion defects.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04163-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Isoproterenol enhances relaxation during ischemia in hypertrophic cardiomyopathy

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


| Name | Title | Affiliation |
|---|---|---|
| James E. Udelson, M.D. | Medical Staff Fellow | CB NHLBI |
| Richard O. Cannon, M.D. | Co-Director Cardiovasc. Diagnosis | CB NHLBI |
| Stephen L. Bacharach, Ph.D. | Imaging Physicist | NM CC |
| Terri F. Rumble | Cardiac Cath. Tech. | CB NHLBI |
| Robert O. Bonow, M.D. | Chief, Nuclear Cardiology | CB NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Nuclear Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)


Many studies have shown that left ventricular relaxation and filling are impaired during myocardial ischemia, even when global pump function may remain intact. These abnormalities may lead to important clinical symptoms such as dyspnea or angina. In this study we sought to determine whether these adverse effects of ischemia on parameters of diastolic performance may be favorably modified, while ischemia was still present. We found that the beta-adrenergic receptor agonist isoproterenol improved parameters of relaxation and filling, despite the simultaneous presence of ischemia in most cases. The results suggest that pharmacologic intervention may alleviate the adverse effects of ischemia on left ventricular relaxation and filling, in an appropriate setting.





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

Project No. Z01 HL 04163-01 CB

Project Description:

Myocardial ischemia impairs left ventricular (LV) relaxation and filling, an important pathophysiologic mechanism in hypertrophic cardiomyopathy (HCM). To determine whether Isoproterenol (I) (known to improve relaxation in isolated muscle) could influence this effect, we assessed simultaneous LV volume (by radionuclide angiography), micromanometer pressure (P), and lactate consumption (LC) in 12 patients with HCM. Pressure volume (PV) relations were studied during pacing stress to induce ischemia and during I infusion to similar heart rates. During I compared to pacing, ischemia was more severe (decreased LC or lactate production) in 9 patients, but peak LVP was higher (204±33 vs 142±21 mmHg $p<.001$), and residual volume (end-systolic/end-diastolic volume) was lower (23±10 vs 29±12%) $p<.01$). Despite ischemia, these changes in load were associated with enhanced diastolic function: I reduced the half time of P decline after peak -dp/dt (33±5 vs 46±9 ms, $p<.01$) and shifted the diastolic PV curve downward and rightward in 10 of 12 patients. Thus, load sensitivity appeared to be present despite ischemia with I, leading to improved LV relaxation and filling. This suggests that in this setting, the adverse effects of ischemia on relaxation may be alleviated by beta adrenergic stimulation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04164-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Effect of lidoflazine on exercise tolerance in microvascular angina

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


| | | |
|---|---|---|
| Richard O. Cannon, III, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| John E. Brush, M.D. | Senior Staff Fellow | CB NHLBI |
| Cynthia M. Tracy, M.D. | Senior Medical Staff Fellow | 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

| 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.)


Many patients with Syndrome X (chest pain despite angiographically normal coronary arteries) have myocardial ischemia due to limited coronary flow reserve (microvascular angina). Although most patients respond symptomatically to calcium channel blockers and nitrates, approximately 25% continue to be significantly limited because of chest pain. In this study, we assessed the hemodynamic and symptomatic efficacy of lidoflazine, a drug that blocks calcium entry into coronary vascular smooth muscle at sites other than the slow channel (the site of activity by calcium channel blockers). To investigate the effect of lidoflazine (240-360 mg/d) on exercise tolerance and hemodynamics, 21 patients with microvascular angina, symptomatic despite prior treatment with calcium channel blockers, underwent graded exercise treadmill testing off all medication, and 3-4 weeks later, on lidoflazine. Compared to baseline, lidoflazine resulted in a 32% increase in total duration of exercise ($p<.005$), and 66% increase in time to onset of chest pain ($p<.005$). A trend toward higher systolic blood pressure - heart rate product (an index of myocardial oxygen demand) may indicate improved coronary blood flow and oxygen delivery during exercise stress in many patients on lidoflazine.




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

Project No. Z01 HL 04164-01 CB

Project Description:

Many pts with Syndrome X (chest pain and normalcoronary arteries) have myocardial ischemia due to limited coronary flow reserve (microvascular angina). Lidoflazine blocks calcium entryinto vascular smooth muscle at sites other than the slow channel, causing vasodilatation. To investigate the effect of lidoflazine (240-360 mg/d) on exercise tolerance and hemodynamics, 21 pts with microvascular angina, symptomatic despite calcium channel blockers, underwent graded tread-mill exercise testing off all medications (control) and 3-4 weeks later, on lidoflazine. Data=mean ± 1 S.D.

| | Control | Lidoflazine | Control | Lidoflazine |
|---|---|---|---|---|
| PRP | 10.2±1.7 | 10.1±2.1 | 21.2±6.2 | 23.1±7.3 |
| Duration (min) | | | 10.0±5.6 | 13.2±5.6* |
| Onset CP (min) | | | 5.8±4.1 | 9.9±6.0* |

*=p<.005 vs peak exercise off all medications,
PRP=systolic blood pressure x heart rate x $10^3$,
CP= chest pain.

Thus, lidoflazine significantly improved effort tolerance and onset of chest pain. The trend toward higher PRP (an index of myocardial oxygen demand) at end-exercise during lidoflazine therapy, although not statistically different from the control study may indicate improved coronary flow and myocardial oxygen delivery during exercise stress in many pts on lidoflazine.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04165-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Effect of lidoflazine on coronary flow reserve

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

| | | |
|---|---|---|
| Richard O. Cannon, III, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| John E. Brush, M.D. | Senior Staff Fellow | 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

| 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.)

We have previously demonstrated that many patients with Syndrome X (chest pain despite angiographically normal coronary arteries) have myocardial ischemia due to limited coronary flow reserve (microvascular angina). Because lidoflazine, a calcium entry blocker, improves chest pain symptoms and effort tolerance in patients with microvascular angina, we studied the effect of this drug on coronary hemodyanmics in 10 patients with microvascular angina. All had been significantly symptomatic on conventional calcium channel blockers and had improved symptomatically on lidoflazine 240-360 mg/d. Compared to measurement of coronary (great cardiac vein) flow off all medications, lidoflazine resulted in improved coronary flow response to pacing stress, even after administration of ergonovine 0.15mg. Further, coronary flow reserve following pharmacologic vasodilation by dipyridamole 0.75 mg/kg was also significantly improved. Thus, lidoflazine results in significant improvement in stress and pharmacologic coronary flow reserve, and suggests that coronary flow reserve in patients with microvascular angina is limited by vasoconstrictor influences related to abnormal calcium fluxes.



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

Project No. Z01 HL 04165-01 CB

Project Description:

We have demonstrated many pts with Syndrome X (chest pain, normal coronary arteries), to have myocardial ischemia due to limited coronary flow reserve, (microvascular angina). Lidoflazine, a calcium entry blocker, improves symptoms in pts with microvascular angina. To investigate the effect of lidoflazine on coronary flow, 10 pts were studied off all medication and after 3 weeks of lidoflazine (240- 360 mg) during 1) pacing stress after ergonovine 0.15mg and 2) dipyridamole 0.75mg/kg. Data= mean ± 1 S.D.

| | | BP | GCVF | CR | MVO2 |
|---|---|---|---|---|---|
| Basal | Control | 105±13 | 69±19 | 1.59±.42 | 7.2±2.0 |
| | Lidoflazine | 93±9 * | 77±13 | 1.25±.23+ | 8.8±2.4 |
| Pacing | Control | 120±14 | 112±46 | 1.23±.45 | 12.4±4.6 |
| | Lidoflazine | 106±18* | 123±44 | .92±.21+ | 14.6±6.8 |
| D | Control | 99±21 | 164±58 | .65±.16 | 9.4±6.9 |
| | Lidoflazine | 93±14 | 220±52* | .46±.15+ | 11.9±6.0+ |

*=$p<.05$, +=$p<.01$ vs control. BP=mean systemic blood pressure (mmHg), GCVF=great cardiac vein flow (ml /min), CR= coronary resistance (BP/GCVF), $MVO_2$= myocardial oxygen consumption (ml $O_2$/min).

Lidoflazine improved coronary flow during pacing stress after ergonovine and following dipyridamole. These results suggest maximal coronary flow is limited in pts with microvascular angina by vasoconstrictor influences related to abnormal calcium fluxes, and that the basic abnormality and resulting symptoms are improved by calcium entry blocking drugs.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04166-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Microvascular angina in hypertensive patients

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

| | | |
|---|---|---|
| John E. Brush, M.D. | Senior Medical Staff Fellow | CB NHLBI |
| Richard O. Cannon,III,M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| William H. Schenke | Cath Lab Technician | CB NHLBI |
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Robert O. Bonow, M.D. | Chiec, Nuclear Cardiology | CB NHLBI |
| Barry J. Maron, M.D. | Senior Investigator | 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

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3 | 2 | 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 studied patients with hypertension, angina pectoris, but with normal coronary angiograms. We hypothesized that these patients have an abnormality of their coronary vasculature which parallels the abnormality in the systemic vasculature causing hypertension, and that this abnormal coronary vasoconstriction results in myocardial ischemia. To prevent the confounding influences of coronary artery disease or left ventricular hypertrophy on estimates of coronary blood flow, patients with angiographic evidence of epicardial coronary artery disease or echocardiographic evidence of left ventricular hypertrophy were excluded. We studied the coronary blood flow in these patients in response to rapid atrial pacing and to intravenous ergonovine. We found that the patients who had reproducible chest pain during atrial pacing had a limitation in their coronary flow reserve. That is their maximal coronary blood flow was 30% less than compared to patients who did not have chest pain during rapid atrial pacing. In addition, it appeared that ergonovine caused further vasoconstriction resulting in decreased coronary blood flow in patients who had chest pain during atrial pacing. Therefore, it appears that patients who have hypertension and angina in normal coronary angiograms, but do not have left ventricular hypertrophy, have an abnormality of the coronary vascular reserve which appears to be a dynamic abnormality. This is the first study which shows an abnormality in coronary vasodilator reserve in hypertensive patients without left ventricular hypertrophy.



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

Project No. Z01 HL 04166-01 CB

Project Description:

We evaluated the coronary vasomotor response to rapid atrial pacing before and after ergonovine (0.15-0.30mg IV) in 19 patients with hypertension, angina, and normal coronary angiograms. Patients with no or mild left ventricular hypertrophy (LVH) were studied. Group A (14 pts) had their typical angina reproduced by pacing. Group B (5 pts) had no symptoms during pacing or ergononovine. Great cardiac vein (GCV) flow was measured at catheterization by thermodilution. Patients had similar base line mean arterial pressure, cardiac index (CI), GCV flow, coronary resistance (CR) and oxygen extraction. Before ergonovine: during pacing, Group A had lower GCV flow (104 vs 148; $p<.01$) and higher CR (1.26 vs .79; $p<.01$) compared to Group B. After ergonovine: at rest, Group A had higher CR (1.66 vs 1.26; $p<.05$); and with pacing, Group A had markedly lower GCV flow (94 vs 167; $p<.001$) and higher CR (1.43 vs .77; $p<.001$) compared to Group B. These differences were not related to LVH. Thus, many patients with hypertension, angina, and normal angiograms have a dynamic abnormality of coronary vasoconstrictor tone at the microvascular level that appears to account for their angina.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04167-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Left ventricular function and drug effects in hypertensive patients

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

| | | |
|---|---|---|
| John E. Brush, Jr., M.D. | Senior Medical Staff Fellow | CB NHLBI |
| James E. Udelson, M.D. | Medical Staff Fellow | CB NHLBI |
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Stephen L. Bacharach, Ph.D. | Imaging Physicist | NM CC |
| Terri F. Rumble | Cath Lab Technician | CB NHLBI |
| Robert O. Bonow, M.D. | Chief, Nuclear Cardiology | CB NHLBI |

COOPERATING UNITS (if any)

Nuclear Medicine, Clinical Center, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3 | 2 | 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.)

Many patients with hypertension have abnormal diastolic relaxation of left ventricular myocardium causing overt congestive heart failure despite normal systolic contractile function. Myocardial cellular calcium overload may contribute to abnormal diastolic function in these patients. Therefore, we studied the effects of a calcium channel antagonist, verapamil, in hypertensive patients and compared the effects of verapamil to equihypotensive doses of nitroprusside. Ventricular function was studied using radionuclide angiography to measure relative LV volume and simultaneously measuring LV pressure using micromanometer-tipped catheters. Patients were atrially pacing to obtain similar heart rates and mean arterial pressure to compare the effects of verapamil and nitroprusside. We found that verapamil had an expected negative ionotropic effect. However, cardiac index rose slightly from 3.1 to 3.3 L/min/$m^2$ with verapamil and this improved left ventricular performance appeared to be due to improved left ventricular filling, evidenced by a downward and rightward shift in the end-diastolic pressure-volume relation. In comparison, nitroprusside decreased left ventricular filling which resulted in a decrease in cardiac index from 3.1 to 2.7 L/min/$m^2$. Therefore, it appears that verapamil is effective in controlling blood pressure while preserving cardiac function due to an improvement in left ventricular filling.

Project No. Z01 HL 04167-01 CB

Project Description:

Left ventricular (LV) diastolic function is impaired in many patients with hypertension. We studied the effects of intravenous verapamil and nitroprusside in 9 symptomatic (angina, normal coronary angiograms) patients with hypertension using simultaneous radionuclide angiography and micromanometer catheters to assess pressure-volume (PV) relations. Compared to baseline, nitro- prusdise decreased end-diastolic volume (EDV, % of B; $p<.05$), and stroke-volume index (SVI, $p<.02$). Compared to nitroprusside at matched mean arterial pressure (MAP) and heart rates (HR), verapamil decreased ejection fraction (EF, $p<.01$) and shifted the end-systolic PV relation down or rightward in all pts, indicating a negative inotropic effect. However, verapamil increased cardiac index (CI, $p<.03$) and LV EDV ($p<.05$) and shifted the end-diastolic PV relation down or rightward in 7/9 pts.

| | HR | MAP | CI | EDV | SVI | EF |
|---|---|---|---|---|---|---|
| B | 107±19 | 127±11 | 3.1±8 | - | 31±11 | 53±10 |
| N | 116±14 | 105±14 | 2.7±.7 | 80 | 24±5 | 59+14 |
| V | 112±16 | 108±12 | 3.3±.7 | 124 | 31±9 | 46±10 |

We conclude 1) in addition to arterial dilatation, the hypotensive effect of nitroprusside is due to a reduction in preload which may impair LV performance and 2) verapamil has negative inotropic effects but maintains LV performance by improving LV filling.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04168-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Probe and fire laser angioplasty using fluorescence atheroma detection

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


| | | |
|---|---|---|
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Louis G. Prevosti, M.D. | Research Associate | CB NHLBI |
| David Y. Lu, M.D. | Medical Staff Fellow | CB NHLBI |
| Paul D. Smith, Ph.D. | Senior Research Fellow | BEIB DRS |
| Robert F. Bonner, Ph.D. | Senior Research Fellow | BEIB DRS |


COOPERATING UNITS (if any)

MCM Laboratories

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

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

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.)


In an effort to develop a laser system which would incorporate plaque recognition with tissue ablation, further developmental efforts utilizing a common fiber technology with fluorescence spectroscopy are being examined. We have helped to develop a prototype dual laser system incorporating a continuous helium cadmium laser (325nm) for fluorescence atheroma detection (probe mode) and a pulsed dye laser (480nm) for tissue ablation. Common optical fibers are used for light excitation of tissue, fluorescence detection, and tissue ablation. After fluorescent sensing, computer-based algorithms direct a fire-no fire signal to the dye laser at 7 repetitions per second for selective atheroma removal. Fluorescence detection of normal and variable composition atheroma were studied in necropsy specimens (n=261) from aorta, coronaries and leg arteries. Although distinctive fluorescence spectra patterns were associated with varying atheroma morphology, an algorithm of normalized spectra ratios differentiated normal sites from abnormal sites reliably ($p<.001$). Probe and fire sequences were tested in normal sites and abnormal sites with 600u fibers demonstrating no laser firing on any normal sites, and correct identification with subsequent tissue ablation in 90% of abnormal sites. Importantly, there were no transmural perforations; firing stopped at the intima-media interface when fluorescence spectra returned to normal after atheroma was removed. We conclude from these preliminary studies that the concept of probe and fire dual laser angioplasty using fluorescence detection can effectively direct selective ablation in vitro and may provide the margin of safety necessary for efficacious in vivo laser angioplasty.




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

Project Description:

A prototype dual laser (L) system has been developed incorporating a continuous HeCd L (325nm, 1.9-3.8mW) for fluorescence (F) atheroma (A) detection (probe mode) and a pulsed dye L (480nm, 2usec pulses) for A ablation (Ab). Common optical fibers are used for light excitation of tissue, F detection, and tissue Ab. After F sensing, computer-based algorithms direct a fire-no fire signal to the dye L at 7 Hz for selective A removal. F detection of normal (N) and variable composition A sites (S) was studied in necropsy specimens (14 pts, n=261) from aorta, coronaries and leg arteries. Although distinctive F spectra patterns were associated with varying A morphology, an algorithm of normalized spectra ratios differentiated NS from AS reliably ($p<.001$). Probe and fire sequences tested in NS (n=20) and AS (n=40) with 600u fibers did not fire on any NS, correctly identified 90% of AS, and the dye L (250 $mJ/mm^2$/pulse) ablated only A. Importantly, there were no transmural perforations; firing stopped at the intima-media interface when F spectra returned to N after A removal. We conclude that this probe and fire dual L system using F detection can effectively direct selective plaque Ab in vitro and may provide the margin of safety necessary for efficacious in vivo L angioplasty.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 04169-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Laser-induced fluorescence plaque detection in patients

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

| Name | Title | Affiliation |
|---|---|---|
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Louis G. Prevosti, M.D. | Reasearch Associate | CB NHLBI |
| Paul D. Smith, Ph.D. | Senior Research Fellow | BEIB DRS |
| Robert F. Bonner, Ph.D. | Senior Research Fellow | BEIB DRS |
| Julie A. Swain, M.D. | Senior Investigator | SB NHLBI |
| Charles L. McIntosh,M.D. | Senior Surgeon | SB NHLBI |

COOPERATING UNITS (if any)

MCM Laboratories

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)

Previously, we have shown that fluorescence spectroscopy can reliably differentiate atheroma from normal sites in necropsy specimens. To determine the feasibility of an in vivo catheter based fluorescence detection system which can be used for clinical laser angioplasty, 25 patients were studied either in the operating room or in the cardiac catheterization laboratory using a 325nm helium cadmium continuous laser excitation source and optical multichannel fluorescence analysis. Both excitation and fluorescence light was transmitted via a common flexible optical fiber bundle to and from remote coronary and aortic sites which were labelled either normal or abnormal based on direct observation or angioscopy. Analyzable spectra (n=124) were obtained from 92% of all sites and required flush fiber-tissue contact. Monomorphic fluorescence spectra from normal sites showed significant variations in peak position among patient subgroups. This inter and intrapatient variability of normal site peaks was far greater than previous necropsy data obtained in our laboratories. Advanced algorithms could distinguish normal sites from abnormal sites in most cases ($p<.005$) but the sensitivity of in vivo abnormal site recognition (67%) was less than in vitro data. We conclude from this preliminary study that fluorescence via optical fibers can be recorded safely and reliably in patients. In vivo tissue optics and other factors may result in higher inter and intrapatient variability of normal site peaks. Fluorescence detection of atheroma in patients, although possible, is somewhat less sensitive when compared to in vitro specimens. However, we believe that the technique of common fiber fluorescence spectroscopy as a means of target recognition can be incorporated within a dual laser computer based system for tissue ablation.

Project Description:

Previously, we have shown that fluorescence (F) spectroscopy can reliably differentiate atheroma (A) from normal (N) sites (S) in necropsy specimens. To determine the feasibility of in vivo catheter-based F detection of A, 25 pts ($51 \pm 13$ yrs) were studied in the operating room (n=12) or cath lab (n=13) using 325 nm excitation (HeCd laser @ 2-2.4mW) and optical multichannel F analysis. Excitation and F light was transmitted via a flexible optical fiber bundle to and from remote coronary (CA) and aorta (Ao) S labelled N or A based on angioscopy or direct observation. Analyzable spectra (n=124) were obtained from 92% of all S and required flush fiber-tissue contact. Monomorphic F spectra from NS (n=83) showed significant variations in peak position among pt subgroups and Ao vs CA. This inter and intrapatient variability of NS peaks was 3X greater than necropsy data. Advanced algorithms could distinguish NS from AS ($p<.005$), but the sensitivity of in vivo AS recognition (67%) was less than in vitro (90%). We conclude: (1) F via optical fibers can be recorded safely and reliably in pts, (2) in vivo tissue optics and other factors create high inter and intrapatient variability of NS peaks, and (3) F detection of A in pts, although possible, is less sensitive when compared to in vitro specimens.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04170-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Responses of canine arterial wall to excimer laser irradiation

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

| | | |
|---|---|---|
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Louis G. Prevosti, M.D. | Research Associate | CB NHLBI |
| Paul D. Smith, Ph.D. | Senior Research Fellow | BEIB DRS |
| Robert F. Bonner, Ph.D. | Senior Research Fellow | BEIB DRS |
| Renu Virmani, M.D. | Chief, Cardiovascular Pathology | CP |

COOPERATING UNITS *(if any)*

Armed Forces Institutes of Pathology

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)*

Although excimer lasers cause precise microablation without surrounding thermal tissue injury, the importance of early and late healing responses must be determined prir to safe therapeutic vascular applications. Therefore, normal canine femoral artery segments were exposed to 308nm excimer laser radiation and animals were sacrificed at 2 hours, 2 days, 10 days, and 6 weeks after initial treatment. Perfusion fixed vascular specimens were analyzed by light, scanning, and transmission electron microscopy. At two hours, after laser treatment the endothelium was desquamated and covered by platelets. A smooth monolayer of axially aligned normal endothelial cells was present without surface organizing thrombus by 10 days. At 6 weeks, there were no aneurysms and no important surface hyperplastic responses. Thus, favorable acute and chronic pathologic responses in vascular tissue suggest that the excimer laser is suitable for clinical laser angioplasty procedures.

Project Description:

Although the Excimer laser (Ex) causes precise microablation without surrounding thermal tissue injury, the importance of early and late healing responses must be determined prior to safe therapeutic vascular applications. In this study, 180 craters (C) of varying depths (15-105u) were formed in 9 normal canine femoral artery segments exposed in vivo to a 308nm Ex @ $36mJ/mm^2$/pulse through 600u silica fibers. After Ex injury, animals were sacrificed (at 2 hours, 10 days, and 6 weeks), arterial segments were in situ perfusion fixed, and Ex sites were analyzed by light, scanning and transmission electron microscopy. At 2 hours, C endothelium was desquamated and covered by platelets and entrapped red blood cells. By 10 days, C depth was unchanged but a smooth monolayer of axially aligned normal endothelial cells was present without surface organizing thrombus. At 6 weeks, there were no aneurysms and only mild surface hyperplastic responses; C were barely visible due to fibrointimal ingrowth consisting of smooth muscle cells in a mucopolysaccharide matrix around a central core of granulation tissue. In this first report of healing to Ex injury of normal canine femoral arteries, we found: 1) early platelet adhesion with rapid reendothelialization and no surface thrombus formation, and 2) late fibrointimal ingrowth without surface damage or aneurysm formation. Thus, these favorable acute and chronic pathologic responses in vascular tissue suggest that the Ex is suitable for clinical laser angioplasty procedures.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04171-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Reduced Surface Thrombegenicity after thermal ablation of plaque

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

| Name | Title | Affiliation |
|---|---|---|
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Louis G. Prevosti, M.D. | Research Associate | CB NHLBI |
| Jeffrey F. Lawrence, M.D. | Research Fellow | CH CC |
| David Y. Lu, M.D. | Medical Staff Fellow | CB NHLBI |
| Paul D. Smith, Ph.D. | Senior Research Fellow | BEIB DRS |
| Robert F. Bonner, Ph.D. | Senior Research Fellow | BEIB DRS |

COOPERATING UNITS *(if any)*

Clinical Hematology, CC

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)*


Thermal and laser angioplasty results in altered endothelial surfaces which may predispose to platelet adhesion and enhanced thrombogenicity. Therefore, to compare acute surface thrombogenicity for different energy sources, atherosclerotic hereditable hyperlipidimic rabbits were exposed to either an ultraviolet pulsed excimer laser, an infrared pulsed erbium-YAG laser, or a catalytic hot tipped catheter to produce comparable longitudinal surface troughs. The segments were perfused with human blood in an anular flow chamber and microscopic morphometry was performed to determine the percent surface area of treated and control sites covered with either adherent platelets or platelets thrombi. Interestingly, thermal ablation using either an erbium-YAG laser or a hot tipped catheter resulted in diminished surface thrombogenicity compared to control sites of excimer laser tissue ablation. These data suggest that precise microablation using ultraviolet pulsed laser sources result in a less favorable thrombogenic surface than thermal tissue ablation.




PHS 6040 (Rev. 1/84)

GPO 914-913

Project No. Z01 HL 04171-01 CB

Project Description:

Surface thrombogenicity (ST) may be a critical factor in laser (L) or hot-tip thermal catheter (HTC) angioplasty procedures. To compare acute ST for different energy sources, 2 cm aortic segments (n=19) from atherosclerotic WHHL rabbits were exposed to: 1) excimer (Ex) L (308nm, 40nsec pulses, 39mJ/mm$^2$/pulse), 2) Erbium-YAG (Er) L (2.94u, 200usec pulse, 75mJ/mm$^2$/pulse), or 3) catalytic HTC (325$^{\circ}$C, 20gm force) to produce 1.5 mm wide longitudinal troughs. The segments were then perfused with citrated human blood in an annular flow chamber. Microscopic morphometry determined the % surface area of treated (T) and control (C) sites covered with adherent platelets (AP) and platelet thrombi (PT).

| | Excimer | | Er:YAG | | Hot-Tip | |
|---|---|---|---|---|---|---|
| | T | C | T | C | T | C |
| AP | 36±8 | 29±5 | 3.4±1.4* | 15±3 | 8±22° | 29±5 |
| PT | 10±7 | 7±3 | 1.5±0.9 | 2.2±.8 | .03±.03° | 9±2 |

*p<.02 vs C. °p<.005 vs C.

Ex L ablation did not alter AP or PT. However, thermal ablation, using either an Er L with minimal thermal damage (~5u) or a HTC with marked thermal damage, resulted in diminished ST. These data suggest 1) Ex L does not change ST and 2) that thermal ablation of artery wall may modify the surface environment such that acute ST is reduced.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04172-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Particulate debris size from excimer and argon laser ablation

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


| | | |
|---|---|---|
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Louis G. Prevosti, M.D. | Research Associate | CB NHLBI |
| John A. Cook | Senior Research Fellow | CB NCI |
| Paul D. Smith, Ph.D. | Senior Research Fellow | BEIB DRS |
| Robert F. Bonner, Ph.D. | Senior Research Fellow | BEIB DRS |


COOPERATING UNITS (if any)

National Cancer Institute

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Cardiology

| 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.)


Feasibility of clinical laser angioplasty will depend upon safe and efficacious removal of obstructing intravascular atheroma. However, different laser sources (visible, infrared, or ultraviolet) and variable lasing parameters (pulsed vs continuous) result in different tissue effects due to different mechanisms of tissue ablation. Since particulate debris after atheroma removal may result in hemodynamically important intraarterial embolic events, it becomes necessary to characterize the photo-products of laser ablation before these techniques can be considered for clinical application. Thus, we determined the size distribution of particular debris after laser ablation of full thickness holes in normal necropsy aorta under saline using a pulsed ultraviolet excimer laser and a continuous argon laser. For both laser sources, more than 80% of particulate debris was less than 3 microns in size, suggesting that the majority of photo-products are too small to cause microvascular obstruction. Interestingly, the excimer laser, despite less thermal tissue injury, caused a 5-10 fold greater number of particles which are greater than 5 microns in size. Since pulsed excimer laser tissue ablation creates shock waves, this mechanism of tissue ablation may result in a larger particle size distribution which may be disadvantageous.





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

Project Description:

We determined the size distribution of particulate debris (PD) after laser (L) ablation of full thickness holes in normal necropsy aorta under saline using both XeCl excimer (Ex) L (308nm, 40 nsec pulse: Ex1-10.5mJ/pulse @ 20Hz; Ex2-5.6mJ/pulse @ 50Hz) and Ar L (488nm, cw: Ar1-4.0W; Ar2-2.3W) light transmitted through 600u silica fibers. PD above a minimum size (~0.5u) was counted in a flow cytometer and evaluated by fluorescence (>514 nm) and $90^{o}$ light scatter. PD histograms were corrected for background and normalized to volume of tissue ablated.

| | # Particles per $mm^3$ of Ablated Tissue | | | | | |
|---|---|---|---|---|---|---|
| | ~.5-1.2u | 1.2-3u | 3-5u | 5-12u | 12-40u | >40u |
| Ex1 | 2,100 | 34,000 | 2,500 | 1,300 | 2,100 | 71 |
| Ex2 | 2,500 | 37,000 | 4,000 | 2,500 | 3,500 | 29 |
| Ar1 | 49,000 | 19,000 | 2,800 | <500 | <500 | 9 |
| Ar2 | 21,000 | 13,000 | 2,000 | <300 | <300 | 5 |

For both L, >80% PD was <3u suggesting that the majority of PD is too small to cause microvascular obstruction. Ex L, despite less thermal tissue injury than with Ar L, causes a 5-10 fold greater number of PD >5u. Ar L ablation results from the continuous vaporization of tissue, whereas the Ex L pulsed ablation creates shock waves. Such shock waves may cause the increased number of larger-sized PD.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04173-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Preferential laser ablation of pigmented atheroma


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

| | | |
|---|---|---|
| Martin B. Leon, M.D. | Co-Director Cardiovascular Diagnosis | CB NHLBI |
| Louis G. Prevosti, M.D. | Research Associate | CB NHLBI |
| Paul D. Smith, Ph.D. | Senior Research Fellow | BEIB DRS |
| Robert F. Bonner, Ph.D. | Senior Research Fellow | BEIB DRS |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Lranch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md

| 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.)


In an effort to help develop and understand new laser sources for clinical laser angioplasty, quantitative evaluations were performed to see if tissue ablation efficiency was dependent upon human plaque composition. Previous reflectance and transmission spectroscopy on variable composition necropsy aorta showed higher absorption at 460-500nm by yellow atheroma. Therefore, two laser sources - a pulsed dye laser (480nm) and a continuous Argon laser (488nm) were used to ablate variable composition necropsy atherosclerotic specimens. For both laser sources, the ablation efficiency increased 5-10 fold for yellow atheroma relative to both white atheroma and normal aorta. Thus, we have demonstrated selective plaque ablation of yellow pigmented atherosclerotic material using laser sources of a specific wavelength (either pulsed or low-power continuous) which correspond to higher tissue absorption characteristics. These data may be important in permiting safe and more efficient atheroma ablation in specific clinical situations during experimental laser angioplasty investigations.





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

Project Description:

The safety and efficacy of laser (L) angioplasty might be improved if L energy were preferentially absorbed by atheroma (A) resulting in selective plaque ablation. Reflectance and transmission spectroscopy was initially performed on variable composition necropsy aorta (Ao) showing higher absorption @ 460-500nm by yellow A. Necropsy Ao was then irradiated via 600 um silica fibers by two L: 1) pulsed dye L (480nm, 20-140 mJ/pulse, 7Hz., 2 usec pulse width), 2) continuous (cw) Argon (Ar) L (488nm, 0.2-2.0 W). For both L, the ablation efficiency at 0.4-0.8W average power increased 5-10 fold for yellow A relative to both white A and normal Ao. The ablation efficiency of the pulsed dye L decreased markedly with reduction of pulse rate and average power. Neither L system was effective at ablating heavily calcified A. The efficiencies of the pulsed L and the cw Ar L were similar at a given average power, which implies a thermal ablation mechanism for both. At higher powers (>2W), the efficiency of Ar L ablation of normal Ao increases and the selectivity of A ablation is diminished. Thus selective thermal ablation of pigmented A may be achieved with either cw Ar L angioplasty systems operating at lower average tissue irradiance (1.4-2.8 $W/mm^2$) than commonly used or with a pulsed dye L @ 480 nm. However, selective ablation is lost at higher average powers and for non-pigmented A.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04174-01-CB

PERIOD COVERED October 1, 1986 to September 30, 1987

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

Promotion of angiogenesis by heparin and non-anticoagulant heparin

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

| | | | |
|---|---|---|---|
| Ellis F. Unger, M.D. | Senior Staff Fellow | CB | NHLBI |
| Cedric D. Sheffield, M.D. | Staff Fellow | CB | NHLBI |
| S. Ward Casscells, M.D. | Senior Staff Fellow | CB | NHLBI |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB | NHLBI |

COOPERATING UNITS (if any) Veterinary Resources Branch, NIH

LAB/BRANCH Cardiology Branch

SECTION Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: 1.2 | PROFESSIONAL: 0.7 | 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.)


Both heparin and non-anticoagulant heparin fragments are known to potentiate angiogenesis in vitro. The purpose of this investigation is to test the ability of heparin and non-anticoagulant heparin fragments to promote intercoronary collaterals in a canine model of myocardial ischemia.

In 24 dogs, the origin of the left anterior descending coronary artery (LAD) will be isolated and an ameroid device will be applied to its proximal portion, effecting gradual occlusion over a 2 to 3 week period. Dogs will be randomly assigned to three treatment arms: group 1 will receive heparin; group 2 will receive an equal quantity of non-anticoagulant heparin fragments; and group 3 will receive only 0.9% saline and serve as the control group. Maximal collateral blood flow to the LAD area will be quantitated with microsphere injections during adenosine induced vasodilatation at various times after surgery, so that the time course and maximal extent of collateral growth can be determined and compared in the three groups.




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

Project No. Z01 HL 04174-01-CB

Project Description:

Both heparin and non-anticoagulant heparin fragments are known to potentiate angiogenesis in vitro. The purpose of this investigation is to test the ability of heparin and non-anticoagulant heparin (KabiVitrum, Stockholm, Sweden) to promote intercoronary collaterals in a canine model of myocardial ischemia.

Twenty-four age and sex-matched hounds will have a left thoracotomy under general anesthesia. The origin of the left anterior descending coronary artery (LAD) will be isolated and an ameroid constrictor (Three Point Products, Toronto, Canada) will be applied to its proximal portion. The dogs will also be instrumented with an inflatable balloon occluder on the LAD and a snare occluder on the left circumflex coronary artery (LCX). Catheters will be placed in the left atrial appendage and internal mammary artery for injection of microspheres and determination of mean arterial pressure, respectively. Dogs will be randomly assigned to three treatment arms: Group 1 will receive heparin at 400 units/kg/day in 0.9% saline. Group 2 will receive an equal quantity of non-anticoagulant heparin fragments on a per weight basis, again in 0.9% saline. Group 3 will receive only 0.9% saline and serve as the control group. A constant infusion pump (Intermedics-Infusaid, Norwood, MA) will be implanted in the abdominal wall for continuous intravenous infusion of the drug. After recovery, radiolabeled microspheres will be injected into the left atrial appendage to determine resting myocardial blood flow. Then, maximal collateral blood flow to the LAD area will be quantitated with microsphere injections during adenosine induced vasodilatation with the LAD balloon occluder inflated at 3 days, 3 weeks, 5 weeks, and 8 weeks after surgery. From these data, the time course and maximal extent of collateral growth will be determined and compared in the three groups.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04175-01-CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

In vivo use of endothelial cell growth factor to effect myocardial angiogenesis

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

| | | | |
|---|---|---|---|
| Ellis F. Unger, M.D. | Senior Staff Fellow | CB | NHLBI |
| Cedric D. Sheffield, M.D. | Staff Fellow | CB | NHLBI |
| S. Ward Casscells, M.D. | Senior Staff Fellow | CB | NHLBI |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB | NHLBI |

COOPERATING UNITS (if any)

Veterinary Resources Branch, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.2 | 0.7 | 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.)


During the last several years, great advances have been made in our understanding of angiogenesis, the study of blood vessel growth and proliferation. Several of the agents which promote this process have been well characterized. One promising agent, endothelial cell growth factor (ECGF), has been synthesized through recombinant techniques.

The purpose of this investigation is to utilize ECGF in a canine model to effect myocardial angiogenesis, and to direct this process to ameliorate myocardial ischemia. In our model, the left anterior descending coronary artery (LAD) of dogs is occluded gradually over a 2 to 3 week period by an ameroid device applied to the proximal vessel. The internal mammary artery (IMA) is implanted into the region normally supplied by the LAD. It is known that collateral vessels develop from the IMA and other sources to supply the territory normally perfused by the LAD. Myocardial blood flow is generally sufficient under rest conditions, but is inadequate under conditions of stress. We have developed a means to quantitate the maximal conductance between the IMA and the LAD vascular bed, assessing myocardial perfusion, the physiologic result of angiogenesis. Ameroids will be applied to the LAD of 16 dogs. The IMA will be implanted in the LAD area. Dogs will be randomly assigned to receive infusions of ECGF or normal saline. After 8 weeks, resting and maximal myocardial blood flow will be quantitated using radiolabeled microspheres. The maximal IMA to LAD bed conductance will be calculated, and comparisons made between groups. In addition, vessels will be examined morphometrically and various hematologic, biochemical, and immunologic parameters will be assessed in the 3 groups, to determine potential adverse effects of ECGF.




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

Project No. Z01 HL 04175-01-CB

Project Description:

During the last several years, great advances have been made in our understanding of angiogenesis, the study of blood vessel growth and proliferation. Several of the agents which promote this process have been well characterized. One promising agent, endothelial cell growth factor (ECGF), has been synthesized through recombinant techniques.

The purpose of this investigation is to utilize ECGF in a canine model to effect myocardial angiogenesis, and to direct this process to ameliorate myocardial ischemia. In our model, the left anterior descending coronary artery (LAD) of dogs is occluded gradually over a 2 to 3 week period by an ameroid device applied to the proximal vessel. The internal mammary artery (IMA) is removed from the inner chest wall and implanted directly into the region normally supplied by the LAD. It is known that collateral vessels develop from the IMA and other sources to supply the territory normally perfused by the LAD. After LAD occlusion, myocardial blood flow is generally sufficient under rest conditions, but is inadequate under conditions of stress. We have developed a means to quantitate the maximal conductance between the IMA and the LAD vascular bed, assessing myocardial perfusion, the physiologic result of angiogenesis.

Ameroids will be applied to the LAD of 16 male foxhounds. The left IMA will be implanted intramyocardially in the LAD area. Dogs will be randomly assigned to receive continuous infusions of ECGF in heparin, heparin alone, or normal saline, directly into the end of the IMA, where vascular connections are developing and angiogenesis is in progress. Drugs will be infused via a constant infusion pump (Intermedics-Infusaid, Norwood, MA) implanted under the abdominal wall. After 8 weeks, resting and maximal myocardial blood flow will be quantitated using radiolabeled microspheres in a terminal experiment. The maximal IMA to LAD bed conductances will be calculated, and comparisons made between the three groups. Vessels will be examined morphometrically as well. Various hematologic, biochemical, and immunologic parameters will be assessed in the 3 groups, to determine potential adverse effects of ECGF.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 04176-01 CB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Internal Mammary Implantation as a Means of Myocardial Revascularization

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

| | | | |
|---|---|---|---|
| Ellis F. Unger, M.D. | Senior Staff Fellow | CB | NHLBI |
| Cedric D. Sheffield, M.D. | Staff Fellow | CB | NHLBI |
| Stephen E. Epstein, M.D. | Chief, Cardiology Branch | CB | NHLBI |

COOPERATING UNITS (if any)

Veterinary Resources Branch, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

| TOTAL MAN-YEARS: 0.8 | PROFESSIONAL: 0.6 | OTHER: 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 a great interest in pursuing methods to more effectively treat patients with coronary artery disease with symptoms refractory to conventional medical therapy, who are not candidates for coronary artery bypass surgery.

A potential approach we investigated was implantation of the internal mammary artery (IMA) into an ischemic region of the left ventricle, to directly revascularize the myocardium independent of the coronary arteries. Long-term results of this procedure, pioneered by Vineberg in the 1940's and widely used before the advent of coronary artery bypass surgery, were controversial. The purpose of this investigation, completed in January, 1987, was to reassess with modern techniques the ability of this operation to provide nutritional flow to ischemic myocardium.

Ameroid devices were applied to the left anterior descending coronary artery (LAD) in 23 dogs, effecting coronary occlusion over 2 to 3 weeks. In 14 dogs, the left IMA was implanted intramyocardially in the LAD zone. The IMA was not implanted in the remaining 9 dogs. After 8 weeks, microspheres were used to quantitate myocardial blood flow to the LAD zone and a control zone under basal conditions and during adenosine-induced vasodilatation, before and after IMA occlusion. Although IMA occlusion caused a detectable fall in LAD zone flow in half of the dogs, no changes in HR, BP, LVEDP, or endo/epi flow ratios were consistently observed. With the IMA patent, there was no difference in mean LAD zone flow between the two groups, either at rest or under conditions of maximal vasodilatation. Thus we concluded that implantation the IMA, while providing some degree of measurable myocardial blood flow in 50% of cases, was not an effective means of myocardial revascularization in this model.




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

Project Description:

We are interested in pursuing methods to more effectively treat patients with coronary artery disease and symptoms refractory to conventional medical therapy, who are not candidates for coronary artery bypass surgery. A potential approach we studied was implantation of the internal mammary artery (IMA) into an ischemic region of the left ventricle. Long-term results of this procedure, pioneered by Vineberg in the 1940's and widely performed before the advent of coronary artery bypass surgery, were controversial.

The purpose of this investigation, completed in January, 1987, was to reassess the efficacy of the Vineberg operation with modern techniques, and determine whether this procedure could potentially be utilized to provide nutritional flow to ischemic myocardium. Ameroid constrictors were applied to the proximal portion of the left anterior descending coronary artery (LAD) in 23 foxhounds. In 14 dogs, the left IMA was implanted intramyocardially in the LAD zone. The IMA was not implanted in the remaining 9 dogs. After 8 weeks, microspheres were used to quantitate myocardial blood flow to the LAD zone (LADZ) and normal zone (NZ) under basal conditions and during adenosine-induced vasodilatation, before and after IMA occlusion. Flow is reported in ml/min/g +/- SEM.

| | BASAL FLOW | | | ADENOSINE FLOW | | |
|---|---|---|---|---|---|---|
| | LADZ | NZ | PERCENT | LADZ | NZ | PERCENT |
| IMA IMPLANTED | 0.54 +/-.07 | 0.69 +/-.08 | 80% +/-4% | 1.32 +/-.13 | 3.03 +/-.31 | 45% +/-3% |
| CONTROLS | 0.54 +/-.06 | 0.70 +/-.07 | 79% +/-8% | 1.31 +/-.16 | 3.32 +/-.51 | 42% +/-4% |

LADZ flow and NZ flow, both at rest and during adenosine, was no different in the 2 groups. IMA occlusion caused a detectable fall in LADZ flow in 7 of 14 dogs with IMA implants; however, no changes in HR, BP, LVEDP, or endo/epi flow ratios were consistently observed. Thus we concluded that implantation of the IMA, while providing some degree of measurable myocardial blood flow in 50% of cases, was not an effective means of myocardial revascularization in this model.

Annual Report of the Laboratory of Cell Biology
National Heart, Lung, and Blood Institute
October 1, 1986 to September 30, 1987

The Laboratory of Cell Biology comprises four Sections with seven independent investigators engaged in different areas of research in cell biochemistry and cell biology. Some of the principal results of the last year are summarized here. These, and other, accomplishments, are described in greater detail in the individual project reports prepared by the investigators.

Biochemistry of Muscle Contraction: Previous work in this Laboratory, under the direction of Drs. Eisenberg and Greene, had led to a model for muscle contraction: during each cycle of ATP hydrolysis, the myosin cross-bridge would alternate between a conformation that binds strongly to actin and a conformation that binds weakly to actin and the cross-bridge is in rapid equilibrium between attached and detached states. On the basis of measurements of the rate of exchange of O-18 between ATP and water, others had concluded that, inconsistent with this model, the two cross-bridges of heavy meromyosin (an experimentally useful derivative of myosin) behaved differently. This was re-investigated this year with results that are fully consonant with the model.

The model proposes that the release of Pi from the active site, following ATP hydrolysis, is the step in the catalytic cycle that is associated with the conformational change of the myosin. Vanadate (Vi) is a useful analogue of Pi in many ATPase reactions. Recently, Drs. Eisenberg and Greene confirmed that Vi inhibits force develop in muscle more than it inhibits the hydrolysis of ATP by the actomyosin complex in vitro. This is not unreasonable. Vi binds to the dissociated myosin-ADP complex. Because the actomyosin complex is under stress in contracting muscle and not when in solution in vitro, it is expected that more myosin-ADP will be dissociated from the actin in the muscle than in vitro. Therefore, more Vi will bind in vivo than in vitro and the inhibition in vivo will be greater than the inhibition in vitro.

The above, and several other experiments, fully support the essentials of the model for muscle contraction. Now that the model appears to have met every serious test that has been proposed, Drs. Greene and Eisenberg consider it relatively unproductive to pursue the subject further at this time and are re-directing their major research activities.

Heat-shock Proteins and Uncoating ATPase: A new area of research has been initiated this year by Drs. Greene and Eisenberg. A group of proteins of Mr about 70,000 increase significantly in concentration when yeast are subjected to heat-shock or other stress. These seem to be related to a protein isolated from brain as an "uncoating ATPase". The function of the uncoating ATPase, as described by Dr. James Rothman and co-workers at Stanford University, is to catalyze the removal of the clathrin coat from the endocytic vesicles known as coated vesicles. These vesicles are formed from coated pits at the cell surface during receptor-mediated endocytosis. It is the aim of the current research to determine the mechanism of action of the uncoating ATPase and to determine the relationship between this enzyme and the homologous heat-shock proteins. Initial studies have shown ATP-dependent removal of clathrin from coated vesicles in the presence of the uncoating

ATPase but the reaction between clathrin and the ATPase appears to be stoichiometric, in the experiments of Eisenberg and Greene, rather than catalytic as reported by Rothman's group.

Non-muscle Myosins and Kinesin: The complete amino acid sequence of *Acanthamoeba* myosin IB heavy chain has been obtained by Dr. Hammer and his associates by sequencing the genomic DNA. The amino-terminal 76 kDa is very similar to the corresponding portion of conventional muscle and non-muscle myosins but the carboxyl terminal 51 kDa is entirely dissimilar. Instead of a sequence predicting a helical coiled-coil structure, as exists in conventional myosins, the COOH-end of myosin IB is rich in glycine, proline and alanine residues. This suggests that myosin IB is the product of a gene which resulted from the fusion of a myosin-like and a non-myosin-like gene. Of 16 introns that interrupt the myosin-like coding region of the gene, 9 are in the same positions as in vertebrate muscle myosin genes in agreement with this hypothesis of the origin of the myosin IB gene.

These sequence results are completely consonant with the continuing biochemical studies on the domain structures of myosin IB and the related myosin IA heavy chains conducted under the direction of Dr. Korn. The ATP-binding site is within 27-kDa of the amino-terminus, probably, by analogy with myosin II, including Glu 109 of myosin IB. The regulatory phosphorylatable serine lies between 38 and 58 kDa of the amino-terminus. One actin-binding site includes amino acid residues on both sides of a protease-susceptible site about 62 kda from the amino-terminus. This actin-binding site is the one involved in the actomyosin ATPase activity. A second actin-binding site resides in the carboxyl-terminal 27 kDa. It is because of the presence of two actin-binding sites that myosins IA and IB can cross-link two actin filaments. Myosin IB can be cleaved at a site 80 kDa from the amino-terminus to a product that seems to be completely analogous to the 95-kDa subfragment-1 from muscle myosin. Both have the actin-activated ATPase activity characteristic of native myosin. The domain distribution described from myosin I is also similar to that for muscle myosin subfragment-1 thus confirming the inferences reached from the sequence homology (about 50%) that this portion of the myosin I heavy chain is myosin-like.

The major difference between myosin I and conventional myosins is that myosin I has a second actin-binding site in its short carboxyl-terminal domain while conventional myosins have a long helical carboxyl-terminal tail. The tail of conventional myosins leads to the formation of bipolar filaments that allow the myosin to cross-link actin filaments so that the cross-bridge cycle can move one actin filament relative to the other. The second actin-binding site in the myosin I heavy chain allows these monomeric myosins to cross-link actin filaments and move one relative to the other in a similar manner.

*Acanthamoeba* myosin II is a conventional myosin with the interesting feature that three phosphorylatable serines that regulate the actin-activated ATPase activity in the globular head are located very far away at the very end of the tail. Dr. Korn and his associates had previously proposed that only filaments of myosin II have actin-activated ATPase activity and that regulation occurs through a conformational change of the filament as a whole. Additional evidence has now been obtained to support both of these hypotheses.

Antibodies have been raised against a synthetic peptide with the sequence of the non-helical tailpiece that contains the regulatory serines and against another synthetic peptide corresponding to the very end of the helical structure that immediately precedes the non-helical tailpiece. Neither antibody inhibits the Ca-ATPase activity of the myosin. The antibody to the non-helical tailpiece has no effect on either filament formation or actin-activated ATPase activity but the antibody to the coiled-coil region inhibits both filament formation and actin-activated ATPase activity. This confirms earlier data that the very end of the coiled-coil segment is essential for formation of bipolar filaments which are essential for actin-activated ATPase activity.

The use of NEM-inactivated myosin II has provided very strong evidence that phosphorylation regulates actin-activated ATPase activity at the level of the filament. NEM (N-ethylmaleimide) adds to the globular head of the myosin II and inactivates its Ca- and actin-activated ATPase activity but does not affect its ability to make bipolar filaments. It has now been found that NEM-unphosphorylated myosin II can activate native phosphorylated myosin II, also when they are present in the same filament, and that NEM-phosphorylated myosin II can inactivate native unphosphorylated myosin II, when they are present in the same copolymer. This must mean that the activity of a myosin II head depends on the level of phosphorylation of the filament in which it resides irrespective of the phosphorylation state of the specific tail to which it is attached.

The structure of myosin II in solution has been assessed through transient electric birefringence measurements. The most likely solution structure for the monomer includes a bend of average angle 110° at a position 36 nm from the carboxyl-tail where the sequence data suggest, and electron microscopic images confirm, there is a bend in the molecule. Monomers form parallel dimers in which the subunits are probably staggered by 28-30 nm and the tails are essentially straight. From these data, a model can be proposed for formation of a filament by association of parallel dimers into anti-parallel tetramers and their association into bipolar filaments. The tetramers would be interdigitated to give the head-to-head spacing of 14-15 nm in the filament. This model gives a filament with a limiting size of 16 monomers and the physical dimensions (length, width and length of bare zone between the two sets of heads) observed by Dr. Pollard, Johns Hopkins University.

Kinesin is the name given to a molecule isolated from squid giant axon and bovine brain that is able to support movement along microtubules. This year, in collaboration with Dr. Bechara Kachar, NINCDS, Dr. Korn and his associates have purified to near homogeneity a similar molecule from Acanthamoeba. They find the purified protein can support the movement of latex beads along microtubules at a very high rate, 3.3 µm/s, and has very high microtubule-stimulated ATPase activity, Vmax=3.4 µmol/min/mg, with a very low Km for microtubules, 0.45 µM.

Actin Polymerization: Last year, Dr. Korn described the purification of a new actin monomer-binding protein, actobindin. This year it has been found that, in addition to binding to actin monomers, actobindin has a property not previously described for any protein. In very low concentration, actobindin inhibits the rate of spontaneous polymerization of actin without

inhibiting either the rate of elongation of existing filaments or the amount of polymerized actin at steady state. All of the results are consistent with the interpretation that nM actobindin inhibits the polymerization of μM actin by inhibiting the nucleation reaction which is the first, and the rate-limiting step, in the polymerization process. A protein such as actobindin would provide a powerful mechanism for inhibiting spontaneous polymerization in the cell while still allowing elongation at existing elongation sites.

Recent work in this Laboratory established that the ATP hydrolysis that accompanies actin polymerization occurs on F-actin subsequent to the polymerization step. Extension of this work by Drs. Carlier and Pantaloni (previous Visiting Scientists in this Laboratory) in France demonstrated the formation of F-actin-ADP-Pi as an intermediate in the conversion of F-actin-ATP to F-actin-ADP. Collaborative experiments between the two laboratories have demonstrated the irreversibility of this reaction by showing the complete absence of exchange between $^{32}Pi$ and ATP and between $^{18}O$-ATP and $H_2O$. This implies that the most of the decrease in free energy occurs at the hydrolysis step itself rather than in the release of products (in contrast to the situation when ATP is hydrolyzed by myosin).

Bioenergetics: Last year, Dr. Hendler described two previously unknown features of heme $a_3$: (1) as the potential was lowered from 450 mV to 200 mV the heme went from a reduced to an oxidized and then to another reduced state which implied that it has two $E_m$ values, one below 450 mV (as previously known) and a high $E_m$ above 750 mV (that had not been previously described). (2) there seemed to be an unusually active, unstable form of heme $a_3$ with a different spectrum than the normal one. Both these observations have been pursued this year.

Electrical titration with the apparatus developed by Dr. Hendler and his collaborators have identified a high $E_m$ for heme $a_3$ with a value of about 780 mV. In contrast to the reduced low $E_m$ form, the reduced high $E_m$ form, does not bind CO. This high $E_m$ state could not have been discovered before the use of electrical titrations because no chemical oxidants of high enough $E_m$ were available to oxidize this state of the heme $a_3$.

Three conditions were established that seem to be needed in vitro to detect the unstable, active form of heme $a_3$. These are the presence of lipoproteins (perhaps to provide an environment similar to that of the membrane environment in which the heme would be in vivo), being held at a voltage >270 mV for at least 1 hour before the reductive titration, and an excess of $K_3Fe(CN)_6$.

Microtubules: Dr. Flavin continues to study the cytoskeleton complex of the Trypanosomatid Crithidia fasciculata. In this organism there is a peripheral corset of microtubules enclosing the cell body and just under the plasma membrane to which it may be linked. The microtubules are maintained at 50-nm spacings by cross-links. Dr. Flavin has identified 3 major proteins associated with this corset apparatus, in addition to tubulin, COP-61, COP-40 and COP-33. In addition, a number of proteins (MAPs) are associated with the cytoplasmic microtubules. None of the COPs induced microtubule polymerization in vitro but crude MAPs did. Microtubule bundles were formed which had

periodic crosslinks at 8.5-nm spacings maintaining separations between microtubules of about 7 to 10 nm. The major component of the crosslinks is thought to be MAP-33. Highly purified COP-33 did not by itself cause polymerization of microtubules but when added to taxol-polymerized microtubules it formed cross-links apparently identical to those formed by MAP-33. COP-33 seems to be a dimer and MAP-33, which has not been purified, is likely to be the same protein.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-HL-00401-21 LCB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Thermodynamic studies of electron and proton affinities of cytochromes

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

| | Name | Title | Affiliation |
|---|---|---|---|
| PI: | Richard W. Hendler | Section Head | LCB, NHLBI |
| Others: | Gurmel S. Sidhu | Visiting Fellow | LCB, NHLBI |

COOPERATING UNITS *(if any)*

Britton Chance and Ali Naqui, Dept. Biochem. and Biophys. University of Pennsylvania, Philadelphia, PA

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Membrane Enzymology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.5 | 1.5 | 0 |

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.)*

Using new redox mediators/buffers and our electrical titration apparatus, we have been able to extend the range of potentiometric titrations up to 900 mV (vs. s.h.e.). With this new capability we have found a high voltage transition of heme a3 with an Em 780 mV. The reduced form of this high potential heme a3 cannot bind CO. Three essential conditions have been established for the formation of the unique "pulsed-like" low potential form of heme a3 with a Soret peak at 429 nm. In the absence of any of the 3 conditions, a different form of low potential heme a3 with a Soret at 448 nm is seen. Preliminary evidence indicates that the pulsed-like form is in a lipoprotein environment, and that it is this form which binds CO in both the oxidized and reduced states. In collaboration with the Johnson Foundation at the University of Pennsylvania, we have used SVD to analyze transient kinetic forms of cytochrome oxidase which arise during the oxidation of the reduced enzyme by O2. We find the same kinds of unique spectra and spectral transitions that we have seen in our equilibrium studies.



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

Our recent published findings on cytochrome oxidase (cyt $aa_3$) obtained with the new techniques for performing potentiometric titrations and data analysis, developed in this laboratory, have raised important questions regarding present theory and previous experimental data. In our last report, we discussed how our findings could be explained by sound thermodynamic analysis. In the present report we provide further experimental support and a rationale for some of our earlier unusual findings. One of these findings was that heme $a_3$ was in the reduced state at the highest attained voltage of 450 mV, and that it became <u>oxidized</u> as the voltage was <u>lowered</u>. This plus the fact that it became re-reduced as the voltage was lowered below 200 mV led us to propose that, because of redox cooperativity, heme $a_3$ had (at least) two $E_m$'s which were controlled by the redox state of another center in the molecule. Proof of the lower $E_m$ was the actual titration that was performed in the lower voltage range. The high $E_m$ was deduced from the fact that heme $a_3$ was in a reduced state at 450 mV. More substantial proof of the high $E_m$ would be the actual titration of the center at high (> 450 mV) voltages. No titrations of any respiratory components have ever been reported in the voltage region above 450 mV. The reason is two-fold. In order to perform a titration in any voltage region, stable redox mediator/buffers with $E_m$ values in the voltage range of interest must be available. Secondly, an oxidant with a sufficiently higher $E_m$ to remove electrons from the mediators with high $E_m$ values must be available. Since other laboratories perform chemical titrations, and no chemical with such a high $E_m$ has proved to be an adequate oxidant no titrations in high voltage regions have been accomplished. We searched the chemical literature and found several possible mediator/buffers having high $E_m$ values. Because we perform electrical oxidations, it is no problem to set as high an effective $E_m$ on the platinum electrode as required to remove electrons from the mediators with high electron affinity. We obtained and tested a variety of the high $E_m$ mediators and found two with the desired properties of stability, ability to exchange electrons with both the enzyme and the platinum electrode, and to be non-denaturing to the enzyme. In this manner we were able to extend the range of the titrations up to 900 mV, which is higher then the $E_m$ of $O_2$, the normal electron acceptor for the enzyme. Using this system we find that heme $a_3$ can be oxidatively titrated with an $E_m$ near 780 mV. Furthermore, when oxidized cyt $aa_3$ is introduced into a solution at 750 mV, heme $a_3$ becomes reduced, which means that its $E_m$ is > 750 mV. It is widely believed that reduced heme $a_3$ binds CO strongly. We find that the reduced form of high $E_m$ heme $a_3$ <u>does not</u> bind CO. It is the reduced form of low $E_m$ heme $a_3$ which binds CO. There were other unresolved questions raised by our low voltage titrations of cyt $aa_3$. We described a previously unseen species of heme $a_3$ in these titrations. The Soret peak was at 429 nm instead of 448 nm and the $\alpha$ peak was at 602 nm instead of 605 nm. This species was seen only in reductive titrations and only when fresh hen egg homogenate was added. We recognized the similarity of the spectrum, and its implied instability, to the properties of an unusually active form of the enzyme called "the pulsed form". The pulsed form of the enzyme is generated by a cycle of oxidation and reduction. In our more recent work we find 3 important prerequisites that must be met in order to generate this pulsed-like form. (1) Egg homogenate must be present. (2) The enzyme must be held at a voltage > 270 mV for about 1 h prior

to the reductive titration. (3) An excess of $K_3Fe(CN)_6$ (over that required for simple mediation) must be present. We have also found that the 429 nm species is not labile. Once formed, it lasts several hours. The requirement for a stage of oxidation followed by reduction is consistent with its possible relation to the pulsed form. The question we have tried to answer is whether our unexpected finding of a form of CO-liganded heme $a_3$ with a "low" $E_m$ value is related to this unusual species of the enzyme. The non-elevation of the $E_m$ of the CO-liganded species implies that the oxidized form has nearly the same affinity for binding as the reduced form, whereas it is widely believed that the reduced form has a much greater affinity for CO-binding. A possible resolution of this apparent contradiction is that the usually seen species of the enzyme is the one examined in the absence of the 3 pre-requisite conditions we have established. What we have found so far is consistent with this idea: When we use the 3 pre-conditions for formation of the low $E_m$, 429 nm species of heme $a_3$, we find that we can both reduce and oxidize the CO-liganded species with an $E_m$ 220 mV. However, when we omit the pre-conditional treatments, we can form the CO-liganded species but we <u>cannot</u> oxidize it in the low voltage region. It therefore seems possible that the form of the enzyme we have observed may represent the enzyme in its natural lipoprotein environment provided by the egg homogenate. The other pre-conditions would serve to generate a pulsed-like form in the lipoprotein matrix. In other laboratories working with the purified enzyme, only the free, non pulsed-like form of the enzyme is seen. Experiments to explore this idea further such as the use of proteoliposomes containing the enzyme are planned.

Collaboration with Ali Naqui and Britton Chance: We initiated a collaboration with the group at the University of Pennsylvania in order to see if our SVD method of analysis, applied to a series of sequential kinetic spectra at extremely low temperature during the oxidation of completely reduced cyt $aa_3$ by $O_2$, would lead to the resolution of the unique kinds of optical spectra which we have seen in equilibrium titrations. In particular, we were interested in the tandem relationship between heme $a_3$ with a Soret band at 429 nm and heme a with a Soret band at 448 nm. We found that as one of these hemes became reduced the other became oxidized and vice-versa. We were also interested in the unique heme $a_3$ spectrum, characteristic of the pulsed form, with a Soret at 429 nm and an $\alpha$ band at 602 nm. In the analysis of 3 sets of data, each of which included kinetic sequences at a variety low temperatures we found the following: (1) The tandem spectrum with a right side up Soret peak at 429 nm and an upside-down Soret at 448 nm. (2) The pulsed-like spectrum with a Soret at 429 nm and an $\alpha$ peak at 602 nm. (3) Peaks at a particular wavelength were seen growing at one point and diminishing at another. One peak at 445 nm showed 4 changes of direction. This behavior is consistent with our ideas of a high degree of redox cooperativity such that a particular redox center can have more than one redox potential, which is dependent upon the redox state of other centers in the molecule. In addition to showing the value of SVD analysis for the resolution of kinetic spectra, these studies showed the appearance of the same kinds of unique spectra during the natural turnover of the enzyme that we have seen in our titration studies.

Publications:

Hendler, R.W., and Reddy, K.V.S.: A new spectral and potentiometric characterization of bovine heart cytochrome _c_ oxidase. 4th EBEC REPORTS, 185 (1986).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL-00409-17 LCB

PERIOD COVERED
October 1, 1986 to September 30, 1987

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)

| | Name | Title | Affiliation |
|---|---|---|---|
| PI: | Evan Eisenberg | Section Head | NHLBI, LCB |
| Others: | John A. Evans | Staff Fellow | NHLBI, LCB |
| | Lois E. Greene | Research Chemist | NHLBI, LCB |
| | José Biosca | Visiting Fellow | NHLBI, LCB |
| | Susan Smith | Visiting Associate | NHLBI, LCB |

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 3.5 | 3.5 | 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.)

We have proposed a simple 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 and is in rapid equilibrium between attached and detached cross-bridge states. In the present study we tested several predictions of this model. First, using O-18 exchange, we tested whether the two myosin heads act independently in their interaction with actin and ATP as predicted by our model. Second, we tested whether the effect of vanadate is different *in vivo* and *in vitro* as has been reported by other workers, and if so, whether this differential effect is consistent with our model. Finally, we used pre-steady-state kinetic studies to determine whether the rate of dissociation of actomyosin by the ATP analogues, AMPPNP and PPi, is similar *in vitro* and *in vivo* as predicted by our model. In all of these studies the results were consistent with our model of muscle contraction.




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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-HL-00506-12 LCB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Acanthamoeba myosins and kinesin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
PI: Edward D. Korn Chief LCB, NHLBI
Others: Joseph P. Albanesi, Staff Fellow, LCB, NHLBI; Mark Atkinson, Visiting Associate, LCB, NHLBI; Hanna Brzeska, Visiting Fellow, LCB, NHLBI; Hisao Fujisaki, Visiting Associate, LCB, NHLBI; Thomas Lynch, Staff Fellow, LCB, NHLBI; Ray Scharff, Chemist, LCB, NHLBI; Toshiyuki Yamakado, Visiting Fellow, LCB, NHLBI; Hidetake Miyata, Visiting Fellow, LCB, NHLBI; Bechara Kachar, Scientist, LNO, NINCDS, Sybren Wijmenga, Visiting Fellow, LCP, NIDDK; Donald Rau, Scientist, LCB, NIDDK

COOPERATING UNITS (if any)
Laboratory of Neuro-Otolalryngology, NINDDS; Laboratory of Chemical Biology, NIDDK; Laboratory of Chemical Physics, NIDDK

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 6.3 | 6.3 | 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.)


Studies of *Acanthamoeba* myosin IA and IB indicate that the ATP-binding site is within 27-kDa of the NH-2-terminus and the regulatory, phosphorylatable serine is between 38 and 58 kDa of the NH-2-terminus. An actin-binding site is about 62-64 kDa from the NH-2-terminus. A second actin-binding site, that is unrelated to ATPase activity but serves to allow myosin I to cross-link actin filaments, is in the 27-kDa COOH-terminal domain. An 80-kDa NH-2-terminal peptide has actin-activated $Mg^{2+}$-ATPase activity but a 62-kDa NH-2-terminal peptide does not, although it is still able to bind to F-actin. This suggests the importance of all or part of the domain between 62 and 80 kDa of the NH-2-terminus. Cleavage between the ATP-binding site and the phosphorylation site (at about 38 kDa from the NH-2-terminus) produces a non-covalent complex in which the ATP and actin-binding sites can still affect each other but phosphorylation of the regulatory serine no longer affects either actin-binding or actin-activated $Mg^{2+}$-ATPase activity.

Additional evidence has been obtained that phosphorylation at the tip of the tail of myosin II regulates actin-activated Mg-2+-ATPase activity of the globular heads by altering the conformation of the filament as a whole. Both phosphorylated and dephosphorylated molecules were shown to be active as long as they are in filaments containing an appropriate level of dephosphorylated tails. Antibodies raised against synthetic peptides corresponding to specific sequences in the tip of the tail have identified a domain that is important for filament formation and consequent enzymatic activity. From electric birefringence studies, it has been possible to deduce the solution structure of myosin II monomers and dimers and propose a possible mechanism of filament formation that leads to a structure consistent with electron microscopic images.

An *Acanthamoeba* kinesin has been purified with very high microtubule-activated Mg-2+-ATPase activity and the ability to translocate latex beads at a high rate from the minus to the plus end of microtubules.




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

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 myosin 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. Recently, a new class of molecules, kinesins, have been described in several cell types (brain, squid giant axon, sea urchin egg). Kinesins seem to be involved in ATP-dependent movements associated with microtubules rather than with actin microfilaments. Studies have been initiated on microtubule-dependent motility in Acanthamoeba.

Major Findings:

Myosin I: Last year, we reported that the 140,000-dalton heavy chain of myosin IA could be cleaved by chymotrypsin into a 112,000-dalton $NH_2$-terminal peptide with the associated 17,000-dalton light chain and a 27,000-dalton COOH-terminal polypeptide. Both peptides bound to F-actin but neither could cross-link actin filaments, as could the native myosin IA. Only the 112,000-dalton peptide had actin-activated $Mg^{2+}$-ATPase activity and it was still regulated by phosphorylation of a single serine residue in that region of the heavy chain. This established that the myosin IA heavy chain has two actin-binding sites, one in an 112,000-dalton $NH_2$-terminal region that resembles muscle myosin subfragment 1, and the other in a novel COOH-terminal region with no analogy to muscle myosins.

This year we extended these studies to show that the ATP-binding site of myosin IA is located in an $NH_2$-terminal 27,000-dalton domain and that the regulatory phosphorylatable serine lies between 38,000 and 58,000 daltons of the $NH_2$-terminus. Limited tryptic hydrolysis cleaves the heavy chain at two sites, 38,000 and 112,000 daltons from the $NH_2$-terminus, producing a 27,000-dalton peptide analogous to that produced by chymotrypsin and a complex between an $NH_2$-terminal 38,000-dalton peptide, a central 74,000-dalton peptide and an undegraded light chain. This complex shows normal ATP-sensitive binding to F-actin and has full ($K^+$,EDTA)-ATPase activity but neither its actin-activated $Mg^{2+}$-ATPase activity nor its affinity for F-actin is affected by phosphorylation, in contrast to native myosin IA. Comparison of the properties of the 112,000-dalton chymotryptic peptide to those of the 38,000/74,000-tryptic complex indicates that cleavage between the ATP site and the phosphorylation site inhibits the effects of phosphorylation on actin binding and actin-activated $Mg^{2+}$-ATPase activity with no effect on the interactions between the ATP and actin-binding sites.

Tryptic digestion of myosin IB heavy chain in the presence of F-actin produces an 80,000-dalton $NH_2$-terminal peptide, which has ATP-sensitive binding to F-actin (but no longer cross-links filaments), and actin-activated $Mg^{2+}$-ATPase activity that is regulated by phosphorylation. In the absence of F-actin, tryptic cleavage produces a 62,000-dalton $NH_2$-terminal peptide and a 68,000-dalton COOH-terminal peptide. The 62,000-dalton peptide still binds to F-actin, still has the phosphorylatable serine and normal ($NH_4^+$,EDTA)-ATPase activity but has no actin-activated $Mg^{2+}$-ATPase activity. These data indicate that F-actin binds myosin IB heavy chain at a site around 62,000 daltons from the $NH_2$-terminus (and protects this site from tryptic cleavage) and that part of all of the domain between 68,000 and 80,000 daltons from the $NH_2$-terminus is important for actin-activated $Mg^{2+}$-ATPase activity. These data are fully consistent with the similar data for myosin IA (above) and the sequence determined with the similar data for myosin IB (see last year) and with the interpretation that the $NH_2$-terminal 80,000-daltons of the unconventional, non-filamentous, myosins IA and IB are highly homologous to the $NH_2$-terminus of conventional, filamentous myosins while the COOH-terminal regions of the unconventional and conventional myosins are totally dissimilar.

The conventional myosins have a single actin-binding site on each $NH_2$-terminal domain but the ability of the COOH-terminal domains to form bipolar filaments allows these molecules to cross-link actin filaments and move one actin filament relative to another. The non-filamentous, unconventional myosins I have a second actin-binding in their COOH-terminal domain which allows single myosin I molecules to cross-link actin filaments and move one relative to the other.

We reported several years ago that myosin I seemed to be preferentially localized in the periphery of the cell and was present in purified preparations of microfilament-plasma membrane complexes. The recent report from Adams and Pollard (Johns Hopkins University) that particles in crude homogenates of Acanthamoeba can translocate along actin filaments, because of myosin I associated with the particles, and our previous observations that pure myosin I can support the movement of latex beads along actin filaments prompted us to re-investigate the possibility that myosin I might be linked to membranes in cells. We have now isolated highly purified plasma membrane-actin filament compleces by an improved procedure and shown them to be enriched in myosin I as determined by ($NH_4^+$,EDTA)-ATPase activity, Coomassie-blue stained electrophoretic gels and immunoblots of electrophoretic gels. Our preliminary evidence suggests that these preparations are enriched for myosin IB, and not myosin IA, and that the myosin IB may be dissociable by 0.6 M KCl + 1 mM MgATP and may be associated with the actin filaments that are attached to the membrane rather than to the membrane itself. These experiments need to be extended before firm conclusions can be reached about the location and possible role of myosin I in the cell.

Myosin II: In contrast to the unconventional myosins I, Acanthamoeba myosin II is a conventional myosin with 2 heavy chains and 2 pairs of light chains. Each heavy chain has a globular head and a helical tail which interacts with the other heavy chain to form a coiled-coil helix

through which the molecules self-associate into bipolar filaments. There are two unusual features about the helical tail, however: (1) there is a non-helical region about one-third of the way in from the COOH-terminus (as deduced from the sequence) and (2) there is a short 29-amino acid non-helical COOH-terminal tailpiece that contains the 3 (or 4) regulatory, phosphorylatable serines. Neither feature has yet been described for other conventional myosins.

Recent electric birefringence data of monomers and dimers have been interpreted to show that the monomer has a bend of 110°, on average, at a position 36 nm from the COOH-terminus which is consistent with the sequence prediction and electron microscopic images. Monomers associate into parallel dimers that most likely are straight molecules with a stagger of 28 nm. With this information it is possible to postulate an assembly process in which two parallel dimers associate into an antiparallel tetramer, and tetramers associate into bipolar filaments. The model is consistent with interdigitating tetramers giving a head-to-head spacing of 14-15 nm, as seen by electron microscopy, and a limiting filament size of about 16 monomers, also as seen by electron microscopy.

Earlier data from this laboratory and Pollard (Johns Hopkins) led to the conclusion that only filaments of myosin II have actin-activated $Mg^{2+}$-ATPase activity and that regulation of this activity by phosphorylation occuurs by a conformational change at the level of the filament. We now propose that phosphorylation may influence the conformation around the potential bend position in the myosin II heavy chain so as to affect the orientation of the globular heads and the way in which they can interact with actin filaments.

Additional evidence for the importance of filaments for actin-activated $Mg^{2+}$-ATPase activity and for the region very near the COOH-terminal for filament formation has come from the use of antibodies raised against synthetic peptides of sequences corresponding to the regions at the very end of the coiled-coil helical portion and the very beginning of the non-helical tailpiece. Antibodies directed to the latter bind to myosin II but have no effect on filament formation or on the actin-activated $Mg^{2+}$-ATPase activity of the filaments. Antibodies to the former bind to the myosin and prevent filament formation and expression of actin-activated $Mg^{2+}$-ATPase activity while having no inhibitory effect on $Ca^{2+}$-ATPase activity.

Previously, we reported that enzymatically inactive phosphorylated myosin II can inactivate enzymatically active dephosphorylated myosin II when both are present in the same filament. It was not possible to tell from these experiments, however, whether the partial activity of the co-polymers was due entirely to the dephosphorylated myosin II molecules or if the phosphorylated myosin II molecules were simultaneously activated. Experiments have now been performed with NEM-inactivated, dephosphorylated myosin II. This molecule is irreversibly enzymatically inactive (both $Ca^{2+}$-ATPase as well as actinactivated $Mg^{2+}$-ATPase activity) because of the derivitization of SH groups in the globular head. It was found that copolymerization of this NEM-dephosphorylated myosin II with phosphorylated myosin II activated the actin-activated $Mg^{2+}$-ATPase

activity of the latter. This is the strongest evidence to date that regulation of myosin II actin-activated $Mg^{2+}$-ATPase activity occurs by conformational changes in the filament. The globular heads of both phosphorylated and dephosphorylated molecules can be active if they are within filaments containing a sufficient fraction of dephosphorylated molecules.

Kinesin: We have obtained a highly purified (but not pure) preparation of Acanthamoeba kinesin which exhibits very high microtubule-stimulated $Mg^{2+}$-ATPase activity ($V_{max}$ about 3.4 µmol $min^{-1}$ $mg^{-1}$; $K_{ATPase}$ about 0.45 µM microtubles) and ability to translocate latex beads along microtubules at a very high rate (about 3.3 µm $s^{-1}$) at a low concentration (0.6 µg $ml^{-1}$). This is the first preparation of a kinesin from any source that has been shown to have both translocating and microtubule-stimulated $Mg^{2+}$-ATPase activity. The rate of translocation is about 5-10 times faster than reported for brain and sea urchin kinesins, the first examples of such molecules, and the microtubule-activated $Mg^{2+}$-ATPase activity is comparable to that previously reported for a brain preparation. As for other kinesins, the Acanthamoeba kinesin supports translocation in only one direction, from the minus to the plus end of microtubules. Although the Acanthamoeba kinesin molecule has not yet been characterized, activity seems to be associated with a complex with a Stokes radius of 8.5 nm comprising three major polypeptides of 134,000, 139,000 and 147,000 daltons.

Publications:

Albanesi, J.P., Lynch, T.J., Fujisaki, H., and Korn, E.D.: Regulation of the actin-activated ATPase activity of Acanthamoeba myosin I by cross-linking actin filaments. J. Biol. Chem. 261: 10445-10449, 1986.

Lynch, T.J., Albanesi, J.P., Korn, E.D., Robinson, E.A., Bowers, B., and Fujisaki, H.: ATPase activities and actin-binding properties of sub-fragments of Acanthamoeba myosin IA. J. Biol. Chem. 261: 17156-17162, 1986.

Albanesi, J.P., Lynch, T.J., Fujisaki, H., and Korn, E.D.: Purification and characterization of an ATP-sensitive actin gelation protein from Acanthamoeba castellanii. J. Biol. Chem. 262: 3404-3408, 1987.

Korn, E.D., Atkinson, M.A.L., Brzeska, H., Hammer, J.A. III, Jung, G., and Lynch, T.J.: Structure-function studies on Acanthamoeba myosins IA, IB and II. J. Cell. Biochem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00514-04 LCB

PERIOD COVERED
October 1, 1986 to September 30, 1987

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 | Research Biologist | LCB, NHLBI |
| Goeh Jung | Visiting Fellow | LCB, NHLBI |
| Jill Horowitz | Guest Researcher | MDA |
| Edward D. Korn | Chief | LCB, NHLBI |

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.2 | 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.)

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. 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. 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 has been of great value in furthering our understanding of the unique structural and functional aspects of the amoeba myosins. 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 study protein structure/function relationships via site-directed mutagenesis of the gene and we can explore the in vivo functions of these myosins by reintroducing the genes back into amoeba.



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

Objectives:

(i) Set up a working system for high level expression of portions of the myosin heavy chain polypeptides in *E. coli*. This approach, coupled with site-directed mutagenesis, deletion analysis, and the biochemical characterization of purified mutant proteins, allows us to directly examine the relationship between important structural elements in the heavy chain polypeptides and myosin function. Two primary candidates for such analysis are the hinge region within the rod domain of myosin II and the unusual glycine- and proline-rich carboxyl terminal domain of myosin IB. With regard to the hinge region, we are interested in defining the role of individual residues in determining the flexibility of this region and, more importantly, the role the hinge might play in filament conformation/enzymatic regulation (using rod/myosin copolymers, for example.) With regard to the unusual C-terminal domain of myosin I, we are interested in examining in detail its interactions with actin and other cellular proteins/structures.

(ii) Continue to develop an expression vector and transformation procedure for amoeba, which hopefully will allow us to use amoeba as an expression system for producing mutant forms of the myosins, generated by protein engineering. This should allow us to conduct structure/function studies wherein the molecular mechanisms underlying enzymatic regulation, actin/myosin interaction and force generation can be directly studied.

(iii) Continue to characterize the two new clones, one of which encodes a myosin II-like molecule and one of which encodes a myosin I-like molecule.

(iv) Continue efforts to identify and isolate genes encoding myosin-I like proteins in other organisms, using myosin I gene fragments encoding portions of the unusual C-terminal domain as heterologous probes.

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, S-1 nuclease mapping, primer extension analysis, expression of amoeba proteins in *E. coli*.

Major Findings:

(i) Myosin II. Performed in depth analysis of the deduced myosin II heavy chain amino acid sequence, including comparison with other myosin sequences by dot matrix analysis and protein structure prediction for the rod domain. With regard to the later, established a collaboration with Dr. Andrew McLachlan (MRC, Cambridge, UK), an expert on rod structure, for further analysis of the rod sequence. In summary we found that the deduced myosin II globular head amino acid sequence shows a high degree of similarity with the globular head sequences of the rat embryonic skeletal muscle and nematode *unc* 54 muscle myosins. By contrast, there is no unique way to align the deduced myosin II rod amino acid sequence with the rod sequences

of these muscle myosins. Nevertheless, the periodicities of hydrophobic and charged residues in the myosin II rod sequence, which dictate the coiled-coil structure of the rod and its associations within the myosin filament, are very similar to those of the muscle myosins. We concluded, based on this information and on the conservation of intron/exon structure between this myosin II gene and characterized vertebrate and invertebrate muscle myosin genes, that this amoeba non-muscle myosin shares with the muscle myosins of vertebrates and invertebrates an ancestral heavy chain gene. The low level of direct sequence similarity between the rod sequences of myosin II and muscle myosins probably reflects a general tolerance for residue changes in the rod domain (as long as the periodicities of hydrophobic and charged residues are largely maintained), the relative evolutionary "ages" of these myosins, and specific differences between the filament properties of myosin II and muscle myosins. In terms of myosin II structure, the single most interesting feature we discovered was the presence of localized flexible point or hinge within the rod-like tail domain. This hinge was identified initially at the sequence level (as a proline-containing perturbation in the repeating coiled-coil sequence), and was confirmed by rotary shadowed electron microscopy of individual myosin II molecules. We hypothesize that this hinge plays a key role in the molecular mechanism by which heavy chain phosphorylation alters filament conformation and, consequently, enzymatic activity. We have initiated attempts to express the rod domain in *E. coli*, which should allow us to study in detail the structure of the flexible region and its role in determining filament conformation and enzymatic activity.

(ii) Myosin IB. Finished the sequence and complete characterization of the genomic clone, which included mapping the 5' and 3' ends of the transcript by S1 nuclease analysis and primer extension analysis (including sequencing of primer extension products). Performed in depth sequence comparisons versus the sequences of conventional myosins, which reveal that the polypeptide is a fusion of myosin-like and non-myosin-like sequences. Specifically, the amino terminal 76 kDa of amino acid sequence is highly similar to the globular head sequences of conventional myosins. By contrast, the remaining 51 kDa of sequence shows no similarity to any portion of conventional myosin sequences, contains regions which are rich in glycine, proline and alanine residues, and lacks the distinctive sequence characteristics of an $\alpha$-helical, coiled-coil structure. We conclude, therefore, that the protein is composed of a myosin globular head fused not to the typical coiled-coil rod-like myosin tail structure but rather to an unusual, glycine-, proline- and alanine-rich carboxyl-terminal domain. These results support the conclusion that filamentous myosin is not required for force generation and provide a new perspective on the structural requirements for myosin function. We also found that of the 16 introns which interrupt the myosin-like coding sequence, the positions of 9 are conserved relative to a vertebrate muscle myosin gene. This has important implications for the antiquity of myosin gene intron/exon structure and further supports our hypothesis that the myosin IB gene was generated by a recombination event in which exons encoding the myosin globular head domain were fused to exons encoding the unusual C-terminal domain. We are currently attempting to isolate cDNA clones for this genomic clone (which is highly interrupted by introns), which is necessary in order to allow expression of the heavy chain in *E. coli* (for structure/function studies

using site-directed mutagenesis).

Related Clones: In attempting to isolate myosin IB cDNA clones from an amoeba cDNA library, we isolated a 2.1 kb cDNA clone which appears to encode a myosin I-like protein. The cDNA clone was mapped and completely sequenced and its deduced amino acid sequence shows strong homology throughout with the myosin IB sequence (spanning the region just above the thiols all the way through the unusual glycine- and proline-rich C-terminal domain to the stop codon). The clone hybridizes to the same size message as do myosin IB gene fragments (3,900 nucleotides). However, the clone does not match exactly the myosin IB sequence (deduced from the gene), nor does it contain the short myosin IA polypeptide sequence. Therefore, we conclude that the clone represents another myosin I isoform (we are checking by S1 nuclease mapping to see if the cDNA clone encodes an alternatively spliced message - perhaps the section of MIA sequence determined at the protein level has been spliced out of this transcript). We screened previously-isolated myosin I-positive genomic clones with this cDNA clone (under high stringency) and obtained a genomic clone which encodes this cDNA. We will sequence this clone in its entirety and will attempt to extend the size of the 2.1 kb cDNA clone for structure/function studies. We are also analyzing another genomic clone which appears to encode a second myosin II heavy chain (MIIB). The MIIB clone hybrid selects a mRNA encoding a 175 kDa polypeptide that comigrates with the myosin II heavy chain. The clone hybridizes to a 5,600 nucleotide mRNA (  500 nucleotides larger than the mRNA recognized by the already-characterized myosin II gene, MIIA). We have sequenced a portion of the MIIB gene and the deduced amino acid sequence for this portion ( 800 amino acids) shows  60% identity with the globular head sequence of myosin IIA. Intron positions in the myosin IIA gene are conserved relative to the MIIA gene. The two important questions we are currently addressing are; (i) does the MIIB polypeptide have a typical coiled-coil rod-like tail (we have yet to identify sequence containing the characteristic heptad repeat of hydrophobic residues, but there is a large region over which this coding sequence is spread), and (ii) is this gene expressed (we will determine if there is an exact match between the 5' flanking sequence of the genomic clone and the sequence of the mRNA, determined by sequencing of primer extension products). If the gene is expressed, we will try to generate myosin II isoform-specific antibodies (using as antigens divergent polypeptides from MIIA and MIIB expressed in *E. coli*). This should allow separation and identification of the two closely related polypeptides, which in turn should allow us to begin to study their different roles in motility, etc.

Publications:

Jung, G., Korn, E.D., and Hammer, J.A. III: The heavy chain of *Acanthamoeba* myosin IB is a fusion of myosin-like and non-myosin-like sequences (1987). Proc. Natl. Acad. Sci. USA (in press)

Hammer, J.A. III, Bowers, B., Paterson, B.M., and Korn, E.D.: Complete nucleotide sequence and deduced polypeptide sequence of a non-muscle myosin heavy chain gene from *Acanthamoeba*: Evidence of a hinge in the rod-like tail (1987). J. Cell Biol. (in press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00515-02 LCB

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Personal Workstation Project for Scientists

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

| | Name | Title | Affiliation |
|---|---|---|---|
| PI: | Richard W. Hendler | Section Head | LCB, NHLBI |
| | Richard I. Shrager | Mathematician | LAS, DCRT |
| Others: | David Songco | Chief, Pers. Work Station Office, DCRT | |
| | Brian Collett | Biophysicist | LPB, NIADDK |

COOPERATING UNITS (if any)
Alan M. Demerle, Chief, Computer Systems Lab, DCRT; Perry Plexico, Chief, Project Development Section, CSL, DCRT; Keith L. Gorlen, James S. Del priore and James Sullivan, CSL, DCRT

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.1 | 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.)

Software has been written for the IBM PC/AT so that it can control an experiment during which a respiratory phase is initiated and data are collected from four electrodes. Other software in connection with this experiment has been developed including a powerful digital filter. A mathematical modelling package (DAL) developed by Brian Collett has been adapted to the IBM PC. A 32-bit co-processor has been obtained and installed in a 16-bit PC. Most of DAL has been adapted to the new 32 bit environment. DAL has been upgraded to handle singular value decomposition.

Background:

Both our group and the group at the Computer Systems Laboratory (CSL) of DCRT are interested in developing a scientific work station based on a microcomputer that will serve the needs of the working scientist as a stand-alone factility for computation and high quality graphics and printed output. Collaboration between the groups involves the exchange of information, bench-mark testing of the two systems, and CSL hardware and engineering support for our PC system.

Progress During the Past Year:

Progress in the development of a 16-bit personal computer of the IBM PC/XT/AT class as a scientific personal work station has proceeded along two lines. RWH has concentrated on the use of the PC to control laboratory experiments and collect data. As described in report number Z01-HL-00418-07-LCB, software has been written which allows the PC to initiate a phase of respiration, collect data from four electrodes, and to display the progress of the experiment in real time using the capabilities of an enhanced graphics adaptor and color monitor. A digital filter program to improve the signal to noise ratio of experimental data has been written. BC has completed the adaptation of his mathematical modelling program (DAL) so that it can run on the PC/XT/AT class machine. Although this is an important step, the limitations of a 16-bit machine for many computational tasks is still a problem. To overcome these limitations, a 32-bit coprocessor (Definicon systems DSI-32) has been obtained. This board expands the available working memory from about 1/2 MB to nearly 2 MB, increases the speed of computation from 2- to 4-fold compared to the IBM PC/AT and eliminates the restrictions of working with only 64 K of data (i.e. 8000 double precision numbers) at a time. BC has installed the DSI-32 in a 16-bit PC and has adapted most of DAL to run in the new environment. In addition, he has incorporated singular value decomposition into the program and is in the final stages of "fine tuning" the graphics capabilities of DAL. It is anticipated that RWH will soon have the completed package for testing and evaluation.

The group in the Computer Systems Laboratory of DCRT has been working independently in evaluating several 32-bit microcomputers. They are also evaluating commerical software packages for statistics, signal processing and mathematical modeling. We plan to run test problems involving large matrices on both their system and our system some time this year.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HL-00516-01 LCB

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

70 kDa Heat Shock Proteins and the Homologous Uncoating ATPase

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


| | | | |
|---|---|---|---|
| PI: | Lois E. Greene | Research Chemist | NHLBI, LCB |
| | Evan Eisenberg | Section Head | NHLBI, LCB |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Cellular Physiology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1 | 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 overall focus of our laboratory is on the function of the 70 kDa heat shock proteins in both normal cellular processes and in the heat shock phenomenon. Although we ultimately plan to investigate the function of these proteins in yeast, we are approaching this question by investigating the relationship of the heat shock proteins to a homologous protein, the uncoating ATPase which appears to be involved in endocytosis in mammalian cells. This protein strips clathrin from clathrin coated vesicles in a process which requires ATP. Our laboratory has isolated coated vesicles, clathrin, clathrin-derived baskets, and the uncoating ATPase from bovine brain. We have developed an assay to quantitatively measure the extent to which the uncoating ATPase removes clathrin from both coated vesicles and clathrin baskets. In contrast to other researchers who have reported that the uncoating ATPase catalytically removes clathrin from these structures, our preliminary results indicate that it may act stoichiometrically. We are currently investigating whether this is indeed the case, and if so, how this stoichiometric effect relates to the ATPase activity of the protein.

Objectives:

Our laboratory has recently changed its research emphasis from studying the kinetics and regulation of the actomyosin ATPase activity to studying the function of the 70 kDa heat shock proteins and a homologous protein with ATPase activity, the uncoating ATPase, which functions in endocytosis by removing clathrin from coated vesicles. As we previously did with actomyosin, we plan to clarifiy the mechanism of action of the uncoating ATPase. We also plan to study, in yeast, the function in both endocytosis and the heat-shock phenomenon of the related 70 kDa heat shock proteins. Our first immediate objective in this research is to determine whether the uncoating ATPase uses ATP hydrolysis to provide energy for the uncoating process, or whether ATP hydrolysis might play a different role, perhaps providing a regulatory cycle as it does with proteins like actin and RAS.

Methods and Major Findings:

During endocytosis, receptors with their bound ligand are transported into the cytoplasm in vesicles which pinch off from the plasma membrane. These vesicles are coated by a cage-like structure primarily composed of the protein, clathrin. Clathrin, which has a subunit composition of three heavy chains of 180 kDa and three light chains of about 30 kDa, has a "triskelion" structure. At low pH (pH 6.0), clathrin, itself, is able to polymerize into baskets, whereas at higher pH (pH 7.0), the assembly of baskets requires small amounts of additional proteins, called assembly proteins. At still higher pH (pH 8.0), clathrin is monomeric, no longer assembling into baskets even in the presence of assembly proteins. It has been shown in the laboratory of Dr. James Rothman, that a 70 kDa-protein dissociates clathrin from coated vesicles and baskets. This protein, which he called the uncoating ATPase, hydrolyzes ATP during the uncoating reaction. At the present time, we are studying the mechanism of action of the uncoating ATPase.

Using the procedure developed by Dr. Rothman, we have purified the uncoating ATPase to greater than 95% purity as judged by SDS gel electrophoresis. We have also purified coated vesicles and by extracting them at pH 8.5, we have obtained a mixture of clathrin and trace amounts of assembly proteins. These proteins were then put in a solution of lower pH, to make clathrin baskets. We have also developed an assay which quantifies the extent of uncoating activity. In this assay the mixture of free clathrin and coated vesicles or baskets is centrifuged and then the free clathrin in the supernatant is measured on SDS gels. Using this assay, we have demonstrated that the uncoating ATPase removes clathrin both from coated vesicles and baskets. We are able to dissociate about 70% of the clathrin present in these structures in a process which requires both ATP and the uncoating ATPase. Surprisingly, however, our preliminary results suggest that the uncoating ATPase appears to be dissociating the clathrin by stoichiometrically binding to it. Whether the clathrin is produced by the uncoating reaction or whether it is added extraneously, the uncoating process seems to stop when clathrin is stoichiometrically bound to the uncoating ATPase. These results disagree with the studies of Rothman and coworkers who reported that the uncoating ATPase acted catalytically rather than stoichiometrically.

Future work:

First, we will continue our investigation of whether the uncoating ATPase removes clathrin from coated vesicles and baskets in a stoichiometric manner. To do this, we will study, over a wide range of concentration, the effect of free clathrin on the uncoating reaction, and will also study the dependence of the uncoating reaction on the concentrations of coated vesicles, baskets, and uncoating ATPase itself. We will also examine the stability of the coated vesicles and baskets under the conditions of our assay to determine the role of ATP in the uncoating reaction. Finally, we will measure the rate of ATP hydrolysis during the uncoating reaction to determine how ATP hydrolysis is related to the actual uncoating activity which occurs during the reaction.

In regard to our project of studying the function of the 70 kDa class of proteins in the heat shock phenomenon, we have entered into a collaboration with Dr. Elisabeth Craig who has characterized 7 genes in yeast which produce proteins homologous to the 70 kDa heat shock proteins. Most of these proteins bind ATP, but their function is unknown. We will be receiving from Dr. Craig strains of mutant yeast which overproduce a particular 70 kDa protein. We will then isolate that protein to determine if it has any uncoating activity. We will also use mutant strains which lack one or more of the 70 kDa proteins to determine if any of them show a reduction in endocytosis using a simple fluorescence assay for endocytosis developed by Dr. Howard Reisman.

ANNUAL REPORT OF THE
LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1986 to September 30, 1987

Research in the Laboratory of cellular Metabolism was for some time 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. Over the past few years, work on the hormone sensitive-adenylate cyclase has evolved to focus on the guanine nucleotide-binding (G) proteins that function in the cyclase and other systems to transmit signals from the exterior of the cell to internal effectors. The objective of this effort is to elucidate mechanisms for control of synthesis, assembly and operation of these ubiquitous regulatory proteins. Major subjects of current studies are $G_o$, a G protein whose physiological role is at present unclear, and several recently recognized, so-called ADP-ribosylation factors that appear to be members of a different family of guanine nucleotide-binding proteins. In addition, work is continuing on regulatory properties of specific cyclic nucleotide phosphodiesterases and calmodulin-activated proteins.

1. Guanine Nucleotide-Binding Proteins in Signal Transduction.

Guanyl nucleotide-binding (G) proteins function in several types of transmembrane signalling systems to couple agonist interaction with cell surface receptors to an intracellular enzymatic response. In adenylate cyclase, inhibitory and stimulatory effects are mediated through $G_i$ and $G_s$, respectively. In the visual excitation complex, $G_t$ (transducin) links the photon receptor rhodopsin to its effector, cGMP phosphodiesterase. $G_o$, which is relatively abundant in brain and is found also in heart, may be involved in regulation of ion channels and phospholipid turnover. These G proteins are heterotrimers of $\alpha$, $\beta$, and $\gamma$ subunits. The $\alpha$ subunits, which are different in each G protein, bind and hydrolyze GTP. They interact with both receptor and effector. We have studied structural, functional, and immunological relationships between $G_s$, $G_i$, $G_t$ and $G_o$. Investigation of other proteins possibly related to the $\alpha$ subunits of the family of G proteins, including the ras oncogene products and a group of so-called ADP-ribosylation factors (described below) is continuing.

With the goal of understanding at the molecular level the mechanism of action and control of synthesis of G protein subunits, we have initially focussed on $G_{o\alpha}$, the $\alpha$ subunit of $G_o$, whose physiological role is unknown. This year, sequencing of a $G_{o\alpha}$ cDNA clone ($\lambda$G09) from a bovine retinal library was completed. $\lambda$G09 has an open reading form encoding 354 amino acids. The nucleotide sequence of $\lambda$G09 and a published partial



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cDNA for rat $G_{o\alpha}$ are 92% identical in coding and 3' noncoding regions. Comparison of deduced amino acid sequences reveals that $G_{o\alpha}$ is most similar to $G_{i\alpha}$ with 73% identity and 82% homology when conservative substitutions are included. Two $G_{t\alpha}$ sequences have 60% and 61% identity and 76% and 78% homology with $G_{o\alpha}$ respectively. $G_{s\alpha}$ is most different with only 34% identity and 50% homology. Marked similarities of sequences of the G proteins, elongation factors, and ras p21 oncogene products are noted in regions believed to be involved in guanine nucleotide-binding and GTP hydrolysis. The λG09 cDNA was used as a probe to screen the retinal library for related cDNAs. Of 400,000 plaques screened, 30 positives were identified and plaque-purified. Restriction analysis of these cDNAs indicates that a number of distinct clones may have been isolated.

A bovine genomic DNA library in Charon 28 was screened using $^{32}P$-labelled restriction fragments of λG09 and 24 positive clones were identified. Ten of the most strongly hybridizing clones were characterized further, five of which were plaque-purified. Restriction digest and hybridization patterns revealed that three of the five clones were identical and hybridized to a different region of the cDNA than did the remaining two clones, which appeared to be, at least in part, different from each other. Many of the inserts have been subcloned in preparation for more detailed restriction analysis and sequencing.

For expression in both E. coli, the λG09 cDNA, starting with the ATG initiation codon and comprising the entire coding sequence with approximately 200 bases of the 3' untranslated region, was ligated into the EcoR1 site of pRC-23. Upon induction, $G_{o\alpha}$ was produced at a level approximating 1-2% of the total cellular protein. On Western blots, the recombinant protein ($rG_{o\alpha}$) reacted with a polyclonal antiserum specific for $G_{o\alpha}$. $rG_{o\alpha}$ also served as a substrate for pertussis toxin catalyzed ADP-ribosylation which was stimulated by addition of transducin βγ subunits providing evidence of an effective interaction of $rG_{o\alpha}$ with βγ. Oligonucleotide site-directed mutagenesis has been initiated to prepare altered forms of $rG_{o\alpha}$ for evaluation of function in reconstituted systems. To analyze expression in mammalian cells, the 1.9 kb full length $G_{o\alpha}$ cDNA was blunt-end ligated into the eukaryotic shuttle vector pSVL and transfected into exponentially growing COS cells. In this system, transient RNA synthesis is directed by the plasmid SV40 transcriptional elements. Initial experiments showed that transcription occurred within 12 h of uptake and continued for up to 80 h.

Myristic acid in amide linkage to the amino-terminal glycine has been found in several cellular proteins. Deduced amino acid sequences of G protein α subunits, which are blocked to Edman degradation, contain a glycine adjacent to the initiator methionine. In addition, $G_{o\alpha}$, $G_{i\alpha}$ and $G_{t\alpha}$ have a serine in position 6 where most myristylated proteins contain serine or

threonine. In collaborative studies, we have shown that $G_{o\alpha}$ contains myristate in hydroxylamine-stable linkage, probably attached to the amino-terminal glycine.

2. <u>ADP-ribosylation of Guanine Nucleotide-Binding Proteins by Bacterial Toxins</u>. Choleragen activates adenylate cyclase by catalyzing, in the presence of NAD, the ADP-ribosylation of $G_{s\alpha}$, the stimulatory GTP-binding protein of the cyclase system. Kahn and Gilman [J. Biol. Chem. 261, 7906-7911 (1986)] identified another GTP-binding protein termed ADP-ribosylation factor (ARF) that stimulated this reaction. They proposed that the toxin substrate is an ARF-$G_{s\alpha}$ complex and that ARF may have a physiological role in regulation of $G_{s\alpha}$ activity. We have found that ARF, purified from bovine brain membranes, enhances not only the ADP-ribosylation of $G_{s\alpha}$, but also $G_{s\alpha}$-independent choleragen-catalyzed reactions. These are the (1) ADP-ribosylation of agmatine, a simple guanidino compound; (2) ADP-ribosylation of several proteins unrelated to $G_{s\alpha}$; and (3) auto-ADP-ribosylation of the toxin $A_1$ peptide. These reactions, as well as the ADP-ribosylation of ARF itself, were stimulated by GTP or stable GTP analogues; GDP and adenine nucleotides were inactive. Our observations are consistent with the conclusion that ARF interacts directly with the A subunit of choleragen in a GTP-dependent fashion thereby enhancing its catalytic activity.

Two soluble ADP-ribosylation factors were purified from bovine brain. These are very similar in size to the membrane ARF (~19 kDa), have the same effects on choleragen activity, exhibit similar GTP requirements and are also ADP-ribosylated by the toxin. One or more of these proteins could be important in the pathological activation of adenylate cyclase by the toxin, which may be viewed as a G protein cascade. Thus, the ARF protein with GTP bound interacts with the $A_1$ peptide of the toxin. This enhances its ability to ADP-ribosylate $G_{s\alpha}$ which, when it has bound GTP, can then activate the cyclase catalytic unit. There is no reason to believe that the ARF proteins have a role in the physiological regulation of $G_{s\alpha}$ or adenylate cyclase. It is unclear whether the apparently very similar forms of ARF are closely related members of a new family of G proteins or whether they differ only in some post-translational modification. Ongoing structural and immunological studies should help to evaluate their possible relationships with the group of G proteins that includes $G_{s\alpha}$ of the family of ras oncogene products and may provide clues to their function.

<u>E. coli</u> heat-labile toxin (LT) which is involved in the pathogenesis of "traveler's diarrhea" is structurally, functionally, and immunologically similar to choleragen. Another E. coli enterotoxin (LT II) also increases adenylate cyclase activity but its mechanism of action had not been defined. In collaborative studies, it was shown that LT II can catalyze the ADP-ribosylation of $G_{s\alpha}$. Thus, although structurally and

immunologically different from LT and choleragen, LT II appears functionally analogous to the other two toxins.

3. Guanine Nucleotide-sensitive $\alpha_1$-Adrenergic Receptor. Agonist binding to $\alpha_1$-adrenergic receptors may be regulated by GTP in a fashion analogous to that observed with the β- and $\alpha_2$-adrenergic receptors, which interact, respectively, with $G_s$ and $G_i$, the stimulatory and inhibitory guanine nucleotide-binding (G) proteins of the adenylate cyclase system. We have begun studies designed to identify the G protein that mediates $\alpha_1$-adrenergic stimulation of phosphatidyl inositol breakdown and release of intracellular calcium. Rat liver membranes incubated with norepinephrine before solubilization with digitonin yielded a soluble hormone-receptor complex from which the release of tightly bound norepinephrine was facilitated by guanyl nucleotides. Binding of the $\alpha_1$-adrenergic receptor antagonist [$^3$H]-prazosin was used to assess guanyl nucleotide-induced release of receptor-bound agonist. Competitive binding studies with adrenergic antagonists, as well as the effects of the antagonists on photo-affinity labeling with a prazosin derivative, were consistent with the presence of an $\alpha_1$-receptor of $M_r$ ~90,000. Effects of GTP, and other nucleotides on norepinephrine release have been defined. This preparation should be useful for further characterization of the receptor-G protein interaction.

4. Regulatory Properties of Cyclic Nucleotide Phosphodiesterases Continuing investigation of the role of a particulate cAMP phosphodiesterase in the regulation of lipolysis by insulin and lipolytic agents was largely focussed in the past year on establishing relationships between lipolysis, cAMP-dependent protein kinase activity and activity of the phosphodiesterase in rat fat cells at varying levels of adenylate cyclase activation or inhibition. Data obtained using the β-adrenergic agonist isoproterenol as a representative lipolytic stimulus provided new evidence that cAMP-mediated kinase-catalyzed phosphorylation is involved in phosphodiesterase activation by lipolytic agents. Insulin activates the particulate cAMP phosphodiesterase through a cAMP-independent mechanism. It can inhibit lipolysis, however, only when cyclase and kinase are less than maximally activated and in a range defined by these studies.

For many years, attempts to purify the insulin-activated phosphodiesterase were unsuccessful. Last year, we succeeded in solubilizing and partially purifying the enzyme from rat adipose tissue membranes in good yield. Inhibitor studies showed that it is very sensitive to cilostamide as well to certain new "cardiotonic" drugs (e.g., milrinone). In continuing collaborative studies, we prepared a derivative of cilostamide coupled to amino-ethylagarose and, using this affinity matrix, have now purified the enzyme ~65,000-fold to apparent homogeneity. Gel filtration and electrophoretic behavior are

consistent with that of a homodimer of ~64 kDa subunits. Based on sensitivity to inhibitors, the purified rat enzyme seems analogous to the insulin-activated enzyme in 3T3-L1 adipocytes, and to the cilostamide-sensitive "low $K_m$" cAMP phosphodiesterases that are present in bovine liver and heart and human platelets. Relationships between these enzymes are being explored. Current studies with the purified hormone-sensitive phosphodiesterase are designed to elucidate mechanisms of activation by lipolytic and antilipolytic agents and to evaluate the possibility raised by our earlier work that a guanine nucleotide-binding (G) protein mediates the effect on the enzyme.

We had previously purified and characterized a so-called cGMP-stimulated phosphodiesterase from bovine liver. Similar soluble enzymes have been found in several other tissues. Recently, investigation of the subcellular distribution of cGMP-stimulated phosphodiesterase in bovine brain revealed that this activity was concentrated in the particulate fraction of grey matter from the cerebral cortex. Both soluble and particulate cGMP-stimulated phosphodiesterases were purified from bovine brain. Both reacted with antibodies prepared against the soluble liver enzyme but differed slightly in size and dramatically in susceptibility to trypsin. Further characterization of these two enzymes is in progress.

5. Regulation of Calmodulin-Activated Phosphodiesterase and Phosphatase. For investigation of mechanisms underlying developmental or regulatory changes in calmodulin-activated phosphodiesterase and phosphatase (calcineurin), isolation of cDNA clones for these proteins was initiated last year. Immunoscreening of cDNA libraries from bovine, murine, and human brain as well as bovine heart resulted in isolation of a phosphodiesterase clone from human brain. The EcoR 1 insert of 135 bp, contains an open reading frame encoding a peptide of ~5 kDa. Isolation of larger cDNAs using this clone as a probe is in progress. Several strongly immunopositive clones for calcineurin have been selected from human and murine brain libraries. Of these, two appear to have large inserts (1600, 1100 bp) that code for fusion proteins of 65 and 40 kDa. One of these fusion proteins interacts strongly with biotinylated calmodulin consistent with the presence of this functional binding domain. While screening for larger phosphodiesterase clones, a rapid procedure employing non-radioactive probes was developed that has proven advantageous for placque hybridization, Southern blot analysis and related hybridization-based methods. Probes labelled with biotinylated dUTP gave signal intensities on dot-blots that were comparable to those observed with 32-P probes and have been effectively used for library screening. This methodology which avoids many of the problems inherent in the use of radioactive probes should have wide applicability.

In collaborative studies, immunocytochemistry was used to

investigate expression of the phosphodiesterase in rat brain during development. Little phosphodiesterase was detectable in brain prior to birth but immediately thereafter discrete clusters of immunopositive cells were observed. Although there was a general concordance with synaptogenesis, there appeared to be instances of substantial expression prior to formation of extensive synaptic contacts. Phosphodiesterase expression appeared to be best correlated with the migration of major neuronal populations, suggesting that the commitment to express precedes the final positioning of the neurons (i.e., establishment of stabilized synaptic contact).

Previous collaborative studies established that the calmodulin-dependent phosphodiesterase from murine testis differed from that in brain. It had a much higher affinity for cAMP and different physical properties, although it reacted with antibodies against the brain enzyme. The subunit size of the testis enzyme was ~66 kDa vs. ~60 kDa for the brain enzyme. Comparison of immunoreactive peptides produced by limited proteolysis with V-8 protease indicated that the enzymes from the two tissues differed considerably. Although the phosphodieterases from brain and testis appear to be distinct polypeptides, similarities in functional regulation by calmodulin, as well as the presence of shared epitopes, suggest that they may arise from related genes or from tissue-specific processing of mRNA.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00614-10 CM

PERIOD COVERED
October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular Regulation of Calmodulin-Dependent Phosphodiesterase and Phosphatase

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: Mauro Giorgi 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); University of Rome (M. Giorgi, R. Geremia).

LAB/BRANCH
Laboratory of Cellular Metabolism, NIH, NHLBI, Bethesda, MD

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
National Institutes of Health, Bethesda, MD 20892

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☐ (a2) Interviews

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

Putative clones for the calmodulin (CaM)-regulated cyclic nucleotide phosphodiesterase (PDE) and protein phosphatase, calcineurin (CN), have been isolated from lambda gt-11 brain libraries and are being characterized. One such clone for PDE, selected by expression vector immunoscreening, contains an EcoRI insert of 135 bp, coding for the fusion protein domain. Because of its small size, it has been necessary to label the insert for use as a probe in obtaining larger cDNAs. A rapid, non-radioactive method for screening phage libraries by placque hybridization was developed for this purpose. Immunocytochemical studies in rat brain indicate that, during development, PDE immunoreactivity appears to be correlated with the migration of certain neuronal populations. This suggests that the commitment to express PDE precedes the final positioning of the neurons (i.e., establishment of stabilized synaptic contact). Biochemical studies of CaM-dependent PDE in mouse testis have shown a novel isozyme that displays higher affinity for cAMP (2 µM vs. 40 µM), and is somewhat larger than the brain enzyme (66 kDa vs. 60 kDa). Since this PDE is intensely immunoreactive with antibody prepared against the brain enzyme, it suggests that they share common epitopes and perhaps similar structural genes. A novel 65 kDa CaM-binding protein (CaM-BP) was observed in murine thymocytes in contrast to that seen in other lymphocyte fractions, (B-,T-cells) where CN was the predominant BP. However, comparison of the proteolytic fragments indicate that the thymus-specific protein may be a precursor form of the phosphatase, consistent with "processing" of this enzyme during lymphocyte development (i.e., into T-cells).

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 events. To isolate and characterize the physical domains of interaction of CaM and its binding proteins. To prepare antibodies and nucleic acid probes specific for these proteins in order to investigate their distribution and biological regulation in tissues and cultured cells.

Methods Employed: Homogenous CaM, biotinylated CaM (Bio-CaM), phosphodiesterase (PDE), calcineurin (CN), and affinity-purified antibody against CN were prepared by previously described methods. Affinity-purified anti-PDE antibody was prepared by chromatography on PDE coupled to CNBr-activated Sepharose. Procedures for immunologic detection in tissue slices and immunoblotting, as well as growth and preparation of murine lymphocytes were as previously reported.

Expression vector immunoscreening was carried out using E. coli 1090r infected with lambda gt-11 libraries as previously described. Stable lysogens of each clone were produced by selective growth at permissive and non-permissive temperature, in phage-infected BNN-103 cells. Fusion proteins produced by the lysogen were detected, after immunoblotting of crude extracts, with a monoclonal mouse anti-beta galactosidase antibody followed by specific alkaline-phosphatase detecting antibodies. In some instances, fusion proteins were purified by affinity chromatography on anti-beta galactosidase coupled to Sepharose. Epitope-selection was carried out as follows: Nitrocellulose filters (150 mm diam.) containing 10 mM IPTG were applied to bacterial lawns seeded with 100,000 phage of an immunopositive clone. After 2.5 h of incubation at 37°, filters were removed, blocked, and washed extensively. The filters, containing bound fusion protein, were incubated with crude antiserum (diluted 50 fold) and subsequently washed. The antibody specifically bound to the filters, was eluted by brief incubation with 0.2 M glycine buffer, pH 2.3 and neutralized.

Phage libraries to be used for placque hybridization were grown on 150 mm dishes for a total of 5-6 hrs. at which time filters were applied to the plate and lifted 5 min later. Bound DNA was denatured and filters were washed and baked. Filters were incubated (30', 37°) with proteinase K (0.1 mg/ml) and washed prior to pre-hybridization for 2 h (50°). Filters were incubated with biotinylated nucleic acid (2 ng/ml) prepared by random-primed labelling, for 8-12 h (50°). After washing of filters, positive placques were detected using avidin or streptavidin-conjugated alkaline phosphatase.

Major Findings: 1) Molecular cloning of cDNAs specific for PDE and CN. Expression vector immunoscreening, initiated last year, was expanded greatly, using cDNA libraries from bovine, murine, and human brain as well as bovine heart. An intensely immunopositive clone, specific for PDE, has been isolated from a human brain library and characterized. The EcoR 1 insert of 135 bp, contains an open reading frame encoding a peptide of ~ 5 kDa. Lysogen extracts of IPTG-induced cultures exhibited 4-fold higher amounts of specific PDE immunoreactivity than did non-induced; as little as 30 ng of fusion protein was detected on Western blots, suggesting an antigenicity comparable to that of the authentic bovine brain protein. The fusion protein was immobilized on nitrocellulose and used to purify PDE antibody from a crude antiserum, in which only a small percentage of antibody was specific for PDE. This technique of "epitope selection" provides strong circumstantial evidence for the authenticity of this clone. In addition, the deduced amino acid sequence of this human brain peptide appears to have a region of high homology with the carboxyl-terminal domain of bovine brain PDE (J. Beavo, University of Washington, personal communication); however, the homology is not retained on either side of this region, suggesting that the carboxyl terminus of these two species may differ considerably. Isolation of larger cDNAs, encoding the highly conserved catalytic region of this enzyme, has been initiated (see section 4, below) to confirm its identity.

Several strongly immunopositive clones for CN have been selected from human and murine brain libraries; of these, two appear to have large inserts (1600, 1100 bp) which code for fusion proteins of 65 and 40 kDa. One of these fusion proteins interacts strongly with biotinylated calmodulin (BioCaM), suggesting that this functional binding region may be retained. A partial deduced amino acid sequence, obtained from the human clone, does not agree with any sequences obtained by protein sequencing.

2) Immunological studies of rat brain CaM-dependent PDE during development and characterization of a novel isoform of PDE in murine testis. In collaboration with Drs. Balaban and Billingsley, recent immunocytochemical studies have examined the expression of PDE during prenatal and early post-natal development in rat brain. Little PDE was detectable in brain prior to birth but immediately after birth, discrete clusters of cells immunopositive for PDE were observed; although there was a general concordance with synaptogenesis, there appeared to be instances of substantial expression prior to formation of extensive synaptic contacts. In some regions, neurons expressed immunoreactivity early and subsequently lost it after reaching their final destination while in other areas, the developing neurons retained PDE expression after their movement was complete. Interestingly, PDE expression appeared to be best correlated with the migration of major neuronal populations, per se.

considerations, use of autoradiography apparatus, half-life of the probe, expense of reagents, etc.) such a non-radioactive detection method should prove highly desirable for general use in molecular biology.

Publications:

Kincaid, R. L., and Vaughan, M.: Molecular and regulatory properties of calmodulin-dependent phosphodiesterase from brain. In Cheung (Ed.): Calcium and Cell Regulation. Academic Press, Inc., 1986, Vol. VI, pp. 43-70.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

Z01 HL 00622-10 CM

PERIOD COVERED
October 1, 1986 through September 30, 1987

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)


| | | | |
|---|---|---|---|
| PI: | Joel Moss, M.D., Ph.D. | Head, Section on Molecular Mechanisms | CM, NHLBI |
| Others: | Jane Halpern, Ph.D. | Staff Fellow | CM, NHLBI |
| | Robert E. West, Ph.D. | Staff Fellow | CM, NHLBI |
| | Tristram Bahnson, M.D. | Md. Staff Fellow | CM, NHLBI |
| | Martha Vaughan, M.D. | Chief | CM, NHLBI |


COOPERATING UNITS (if any)
Randall Holmes, Dept. of Microbiology, USUHS, Bethesda, MD

LAB/BRANCH
Laboratory of Cellular Metabolism, NIH, NHLBI, Bethesda, MD

SECTION
Molecular Mechanisms

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


1) Regulation of the hormone-sensitive adenylate cyclase is mediated by two guanine nucleotide-binding (G) proteins, stimulation through Gs, inhibition through Gi. In the visual excitation system, a similar G protein, Gt or transducin, couples the photon receptor rhodopsin to a cyclic GMP phosphodiesterase. These G proteins are heterotrimers of α, β, and γ subunits. The α subunits bind guanine nucleotide and hydrolyze GTP. The βγ subunits facilitate coupling of α to receptor. To determine the function of Gγ, a mouse anti-Gtγ monoclonal antibody (2H3) was prepared. 2H3 was specific for Gtγ and did not recognize Gγ from liver or brain. Interaction of 2H3 with Gtαβγ appeared to facilitate the dissociation of the G protein into its α and βγ components, as monitored by pertussis toxin-catalyzed ADP-ribosylation (which favors the heterotrimer and is inhibited by 2H3) and immunoprecipitation (in which βγ was precipitated but not α). In the presence of receptor, the effect of 2H3 was diminished, consistent with the hypothesis that a domain on Gtγ is masked in the rhodopsin·Gt complex. The studies, thus, support a role of Gγ in a protein-receptor coupling. 2) Bacterial toxins, such as pertussis and cholera toxins, exert their effects of cells through the ADP-ribosylation of G proteins; this modification leads to altered function. In the case of cholera toxin, ADP-ribosylation of Gsα results in adenylate cyclase activation. A novel toxin from Escherichia coli (LT-II) appeared to catalyze the ADP-ribosylation of Gsα, leading to activation of adenylate cyclase, suggesting that LT-II and cholera toxin, two nonhomologous and immunologically different toxins, share a common mechanism of action.

Project Description

Objectives: To define mechanisms for regulation of cAMP and cGMP metabolism by hormones, drugs, neurotransmitters, and bacterial toxins. cAMP synthesis is controlled through the hormone-sensitive, membrane-associated adenylate cyclase system. The cyclase complex consists, at a minimum, of inhibitory and stimulatory hormone receptors linked through different guanine nucleotide-binding regulatory (G) proteins, termed $G_i$ and $G_s$ for those involved in inhibition and stimulation, respectively, to a catalytic unit. A functionally analogous system is present in the visual excitation complex, where the photon receptor rhodopsin signals through a G protein $G_t$ to activate a cGMP phosphodiesterase. $G_s$, $G_i$ and $G_t$ are heterotrimers of $\alpha$, $\beta$, and $\gamma$ subunits. The $\alpha$ subunits bind guanine nucleotide and have intrinsic GTPase activity; hydrolysis of bound GTP to GDP results in inactivation. GTP-GDP exchange and GTPase activity are enhanced by agonist-receptor complex. Pertussis toxin, an etiologic agent in whooping cough, catalyzes the ADP-ribosylation of $G_{i\alpha}$, $G_{t\alpha}$ and other $G_\alpha$ proteins, leading to stabilization of $G_{\alpha\beta\gamma}$ uncoupled from receptor with resultant loss of receptor-stimulated GTPase activity. To study the function of the $\gamma$ subunit in the association of $G_{\alpha\beta\gamma}$ and the interaction of G protein with receptor, a monoclonal antibody against $G_{t\gamma}$ was used.

Choleragen (cholera toxin) (CT) and E. coli heat-labile toxin, (LT), agents involved in the pathogenesis of cholera and "traveler's diarrhea," respectively, activate cyclase by ADP-ribosylating $G_{s\alpha}$. ADP-ribosylated $G_{s\alpha}$ exhibits increased sensitivity to GTP due to decreased GTPase activity. An E. coli enterotoxin termed LT-II immunologically different from CT and LT, increases adenylate cyclase activity, but its mechanism of action has not been defined. To determine whether it is an ADP-ribosyltransferase that can modify $G_\alpha$ proteins, cultured human fibroblasts were intoxicated, and purified LT-II was investigated enzymologically.

Methods Employed: (1) Assays: NAD glycohydrolase, ADP-ribosyltransferase, adenylate cyclase, GTPase, and ADP-ribosylation assays were performed by modifications of methods developed in this laboratory. cAMP levels were determined by a commercial radioimmunoassay kit. (2) Protein and Antibody Preparation and Purification: E. coli enterotoxin was purified by published methods. A murine monoclonal antibody specific for $G_{t\gamma}$ by ELISA and immunoblot was prepared as described previously.

Major Findings: 1) Immunological mapping of functional domains in G proteins: A monoclonal antibody against $G_{t\gamma}$ (2H3) did

not cross-react with γ subunits of G proteins purified from rabbit liver or bovine brain, consistent with other evidence that they are different from $G_{t\gamma}$. To study γ subunit function, we examined effects of 2H3 on G protein subunit interaction and G protein-receptor coupling. In the absence of the receptor rhodopsin, 2H3 inhibited pertussis toxin-catalyzed ADP-ribosylation of $G_{t\alpha}$ in a dose-dependent manner; ADP-ribosylation requires formation of a $G_{\alpha\beta\gamma}$ complex. Complete inhibition of this reaction occurred at ~ 1:1 molar ratio of transducin to 2H3. No inhibition was apparent when rhodopsin was present. Presumably 2H3, in the absence but not in the presence of rhodopsin, can inhibit pertussis toxin-catalyzed ADP-ribosylation by preventing the association of α and βγ subunits to form the preferred substrate αβγ. In agreement with the hypothesis that 2H3 promotes dissociation was the finding that 2H3 immunoprecipitated only βγ from holotransducin. A role for the γ subunit in receptor-G protein coupling was supported by the observation that 2H3 inhibited rhodopsin-stimulated $G_t$ GTPase activity, presumably by interfering with formation or function of the rhodopsin-transducin complex.

2) Activation of adenylate cyclase in human fibroblasts by LT-II: Fibroblasts incubated with LT-II had a four-fold increase in adenylate cyclase activity. In membranes, activation of cyclase was enhanced by NAD, GTP, and dithiothreitol; the requirements for activation of cyclase by LT-II are similar to those observed with cholera toxin (CT), an NAD:arginine ADP-ribosyltransferase. In fibroblast membranes, similar proteins were [$^{32}$P]ADP-ribosylated by LT II and CT. The molecular weights of the primary [$^{32}$P]ADP-ribosylated proteins were consistent with their being α subunits of $G_s$, the stimulatory regulatory guanine nucleotide-binding components of adenylate cyclase. LT-II and CT-specific labelling was significantly decreased in membranes prepared from fibroblasts preincubated with either LT-II or CT. In the absence of cellular components, LT-II catalyzed the ADP-ribosylation of agmatine, a simple guanidino compound. These data are compatible with the conclusion that LT-II is an ADP-ribosyltransferase that activates adenylate cyclase by modification of $G_{s\alpha}$.

Significance to Biomedical Research and the Program of the Institute:

The pulmonary and cardiovascular systems are affected under physiological and pathological conditions by agents such as hormones and toxins. The lung, in particular, is exposed to numerous bacterial and toxic agents. A substantial number of these exert their effects by altering the levels and localization of cyclic nucleotides within the cell. Some bacterial toxins, e.g., pertussis toxin, an etiologic agent in whooping cough, exert their effects on cyclic nucleotides by catalyzing the covalent modification of critical regulatory proteins, thereby perturbing their function. A number of therapeutic drugs that

override aberrant physiological control to the benefit of the patient have been designed to interact with specific cellular receptors, and thus alter cyclic nucleotide levels. By using cultured cell model systems and purified proteins, it may be possible to define mechanisms that regulate these pathways which are critical for the normal function, responsiveness, and complex interaction of many types of cells in the pulmonary and cardiovascular systems.

Proposed Course: (1) To define further the regulation of guanine nucleotide-binding proteins by hormones, toxins, drugs and neurotransmitters; (2) To study the regulation of ADP-ribosylation pathways 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 00627-09 CM

PERIOD COVERED

October 1, 1986 through September 30, 1987

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)


| | Name | Title | Affiliation |
|---|---|---|---|
| PI: | Su-Chen Tsai, Ph.D. | Research Chemist | CM, NHLBI |
| Others: | Masatoshi Noda, Ph.D. | Visiting Fellow | CM, NHLBI |
| | Barbara C. Kunz, Ph.D. | Visiting Fellow | CM, NHLBI |
| | David Bobak, M.D. | Sr. Staff Fellow | CM, NHLBI |
| | M. Michael Bliziotes, M.D. | Sr. Staff Fellow | CM, NHLBI |
| | Kimberly Muczynski, M.D., Ph.D. | Md. Staff Fellow | CM, NHLBI |
| | Kim Williamson, Ph.D. | Staff Fellow | CM, NHLBI |
| | Joel Moss, M.D., Ph.D. | Head, Sec. Mol. Mech. | CM, NHLBI |
| COOPERATING UNITS (if any) | Martha Vaughan, M.D. | Chief | CM, NHLBI |


LAB/BRANCH

Laboratory of Cellular Metabolism, NIH, NHLBI, Bethesda, MD

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

National Institutes of Health, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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| 5.3 | 4.3 | 1.0 |

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☐ (a1) Minors

☐ (a2) Interviews

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


Choleragen activates adenylate cyclase by catalyzing, in the presence of NAD, the ADP-ribosylation of Gsα, the stimulatory GTP-binding protein of the cyclase system. Kahn and Gilman [J. Biol. Chem. 261, 7906-7911 (1986)] identified another GTP-binding protein termed ADP-ribosylation factor (ARF) that stimulated this reaction. It was proposed that the toxin substrate is an ARF-Gsα complex and that ARF may have a physiological role in regulation of Gsα activity. ARF, purified from bovine brain membranes, enhanced not only the ADP-ribosylation of Gsα, but also Gsα-independent choleragen-catalyzed reactions. These are the (1) ADP-ribosylation of agmatine, a low molecular weight guanidino compound; (2) ADP-ribosylation of several proteins unrelated to Gsα; and (3) auto-ADP-ribosylation of the toxin A1 peptide. These reactions, as well as the ADP-ribosylation of ARF itself, were stimulated by GTP or stable GTP analogues such as guanyl-5'-yl imido-βγ-diphosphate and guanosine 5'-O-(3-thio-triphosphate); GDP and guanosine-5'-O-(2-thiodiphosphate) were inactive. These observations are consistent with the conclusion that ARF interacts directly with the A subunit of choleragen in a GTP-dependent fashion thereby enhancing catalytic activity manifest as transfer of ADP-ribose to Gsα and other proteins, to the toxin A1 peptide, or to agmatine. We have also purified two other soluble factors that enhanced the ability of choleragen to ADP-ribosylate Gsα. Each exhibited properties similar to ARF leading to the speculation that a family of ARF-like proteins may exist in animal cells.

Project Description

Objectives: To define the regulation of the hormone-sensitive adenylate cyclase system. The hormone-sensitive adenylate cyclase system, which is critical to the regulation of cellular processes by hormones, toxins and drugs, consists of stimulatory and inhibitory receptors linked through different guanine nucleotide-binding (G) regulatory proteins to a catalytic unit. These G proteins are heterotrimers consisting of α, β, and γ subunits. $G_{s\alpha}$, the G protein involved in stimulation of adenylate cyclase, is ADP-ribosylated and thus activated by cholera toxin. Toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ was enhanced by a GTP-binding protein known as ARF. It was proposed (Kahn and Gilman (1986) J. Biol. Chem. 261, 7906-7911) that ARF increases ADP-ribosylation of $G_{s\alpha}$ by forming an ARF·$G_{s\alpha}$ complex. In contrast, preliminary evidence in our laboratory was consistent with an effect of ARF on toxin, not $G_{s\alpha}$. To investigate its mechanism of action, the protein was purified and its properties determined.

Methods Employed: ADP-ribosylation factor (mARF) from bovine brain membranes was purified by published methods with some modifications. ADP-ribosylation factors (sARF-I and sARF-II) from bovine brain soluble fraction were purified by our methods. Choleragen-catalyzed $^{32}$P-ADP-ribosylation and NAD:agmatine ADP-ribosyltransferase activity of choleragen A subunit were assayed with our published procedures.

Major Findings:
(1) Kahn and Gilman proposed that a membrane-derived ARF (mARF), in the presence of GTP, enhances the choleragen-catalyzed ADP-ribosylation of $G_{s\alpha}$ by forming an ARF-$G_{s\alpha}$ complex thereby increasing the availability of the arginine in $G_{s\alpha}$ that is modified by the toxin. To determine whether the effect of ARF was directly on the toxin rather than on $G_{s\alpha}$, we took advantage of the fact that cholera toxin catalyzes several ADP-ribosylation reactions independent of $G_{s\alpha}$. We found that, in addition to stimulating choleragen-catalyzed ADP-ribosylation of $G_{s\alpha}$, ARF enhances the auto-ADP-ribosylation of the toxin $A_1$ peptide, the ADP-ribosylation of agmatine, and the hydrolysis of NAD to ADP-ribose and nicotinamide. All the choleragen-catalyzed reactions were enhanced by ARF in a GTP-dependent manner; stable GTP analogues such as guanyl-5'-yl imido-βγ-diphosphate and guanosine-5'-O-(3-thio-triphosphate) were also effective whereas GDP, guanosine-5'-O-(2-thiodiphosphate), and adenyl-5'-yl- imido βγ-diphosphate were inactive. Our data demonstrated that ARF can interact with the toxin independent of $G_{s\alpha}$ and in doing so increases the catalytic activity of the $A_1$ peptide in three $G_{s\alpha}$-independent reactions.

Publications:

Tsai, S-C., Adamik, R., Kanaho, Y., Halpern, J., and Moss, J.: Immunological and biochemical differentiation of guanyl nucleotide-binding proteins: interaction of $G_{o\alpha}$ with rhodopsin, anti-$G_{o\alpha}$ polyclonal antibodies and a monoclonal antibody against transducin α-subunit and $G_{i\alpha}$. Biochemistry, in press.

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Noda, M., Tsai, S-C., Adamik, R., Chang, P.P., Kunz, B.C., Moss, J., and Vaughan, M.: Activation of the NAD glycohydrolase, NAD:agmatine and NAD:$G_{s\alpha}$ ADP-ribosyltransferase and auto-ADP-ribosylation activities of choleragen by guanyl nucleotide and soluble proteins purified from bovine brain. In Jacobson, M. and Jacobson, E. (Eds.): Niacin Nutrition, ADP-Ribosylation and Cancer. New York, Springer-Verlag, 1987, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00634-07 CM

PERIOD COVERED
October 1, 1986 through September 30 , 1987

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: Seiko Murashima, M.D., Ph.D. Visiting Fellow CM, NHLBI

Others: Vincent C. Manganiello, M.D. Ph.D. Head, Section on Biochemical Physiology CM, NHLBI


COOPERATING UNITS *(if any)*

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Laboratory of Cellular Metabolism, NIH, NHLBI, Bethesda, MD

SECTION
Biochemical Physiology

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National Institutes of Health, Bethesda, MD 20892

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☐ (a1) Minors

☐ (a2) Interviews

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


The subcellular distribution of cGMP-stimulated and calmodulin-sensitive cyclic nucleotide phosphodiesterases (PDEs) in bovine brain was studied. Although the cGMP-stimulated PDE has been purified to apparent homogeneity from supernatants of bovine hepatic, cardiac and adrenal tissues, in bovine brain this PDE is concentrated in particulate fraction of grey matter from cerebral cortex. Washed particulate fractions exhibit little apparent calmodulin-sensitive PDE activity, but in the presence of detergent considerable "latent' calmodulin-sensitive PDE activity was expressed. The detergent-solubilized, particulate cGMP-stimulated PDE was purified via cyclic nucleotide affinity chromatography and exhibits in SDS-PAGE an Mr slightly greater than the soluble form of the enzyme.

Project Description:

Objectives: To understand the characteristics of the cGMP-stimulated phosphodiesterase (PDE) and its regulation and importance in intact cells.

Methods Employed: Portions of bovine brain cerebellum and cerebral cortex were dissected; grey and white matter were homogenized and centrifuged (100,000 x g, 1 h). Particulate fractions were resuspended and homogenized in salt-free buffer or buffer containing 500 mM NaCl, and recentrifuged. Washed pellets were sonicated or homogenized in buffer containing Lubrol (up to 1%), kept overnight at 0-5°C, and centrifuged (100,000 x g, 1 h). Material remaining in the supernatant was referred to as "solublilzed". Solubilized cGMP-stimulated PDE was purified by sequential chromatography on cAMP-agarose and cGMP-agarose. The cGMP-stimulated PDE bound to cGMP-agarose, and after several contaminants were eluted with 10 µM cAMP, the PDE was eluted with 1 mM cAMP and concentrated on DEAE-Sepharose. Enzyme purity was assessed via SDS-PAGE (10% gels) and by Western immunoblots with antibody raised against the purified liver cGMP-stimulated cAMP PDE.

Major Findings: In bovine brain, the cGMP-stimulated PDE is concentrated in grey matter from cerebral cortex. In homogenates of cortex, ~ 70-75% of this PDE is found in association with washed (hypotonic buffer or buffer containing 500 mM NaCl-1 mM EDTA) particulate (100,000 x g, 60 min) fractions; these fractions exhibit little apparent calmodulin-sensitive PDE activity. Purified liver soluble cGMP-stimulated PDE was incubated with several non-ionic and zwitterionic detergents. CHAPS inhibited both basal and cGMP-stimulated cAMP hydrolytic activity. Lubrol and other non-ionic detergents did not inhibit cAMP hydrolysis and inhibited cGMP-stimulated activity at detergent concentrations greater than 1%.

Brain particulate fractions were therefore solubilized in buffer containing 1% Lubrol. In the presence of Lubrol, particulate cGMP-stimulated activity was increased no more than 2-fold, but calmodulin-sensitive PDE activity was increased at least 5-6 fold, indicating that much more of the calmodulin-sensitive activity was in a "latent" form. Detergent extracted fractions were centrifuged at 100,000 x g for 60 min; virtually all of the cGMP-stimulated and calmodulin-sensitive PDE activity was effectively solubilized. A cAMP PDE activity was not solubilized; this particulate form was very sensitive to inhibition by Rolipram, not by cilostamide. The solubilized cGMP-stimulated and calmodulin-sensitive forms were separated by chromatography on DEAE. cGMP-stimulated PDE forms were purified from both brain soluble and particulate fractions by sequential chromatography on cAMP and cGMP-agarose. Analysis on SDS-PAGE

and by Western immunoblots indicated that the apparent $M_r$ of the particulate form was slightly greater than that of the soluble form isolated from brain or liver.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the characteristics and regulatory properties of cyclic nucleotide phosphodiesterases is important for understanding mechanisms that regulate effector-mediated physiological and pathological processes in mammalian cells. This particular phosphodiesterase may be of importance in regulation of cyclic nucleotide metabolism in several tissues including cardiac and adrenal tissue and may be especially involved in mechanisms whereby atriopeptins and cGMP regulate cAMP content in certain cells.

Proposed Course: To compare characteristics of particulate and soluble forms of the cGMP-stimulated PDE; to identify and characterize cDNA clones for the PDE with the aim of learning more about the structure and function of this PDE and its regulation and function in cells.

Publications:

Wada, H., Osborne, Jr., J.C., and Manganiello, V.C.: Effects of temperature on allosteric and catalytic properties of the cGMP-stimulated cyclic nucleotide phosphodiesterase from calf liver. J. Biol. Chem. 262:5139-5144, 1987.

Wada, H., Manganiello, V.C., and Osborne, Jr., J.C.: Analysis of kinetics of cAMP hydrolysis by the cGMP-stimulated cyclic nucleotide phosphodiesterase. J. Biol. Chem., in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00636-06 CM

PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Particulate PDE in the Regulation of Lipolysis by Insulin and Lipolytic Agents

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


| | | | |
|---|---|---|---|
| PI: | Carolyn Smith, Ph.D. | PRAT Fellow | CM, NHLBI |
| Others: | Vincent Manganiello, M.D., Ph.D. | Head, Section on Biochemical Physiology | CM, NHLBI |
| | Martha Vaughan, M.D. | Chief | CM, NHLBI |


COOPERATING UNITS (if any)

Eva Degerman and Per Belfrage, Department Physiological Chemistry, University of Lund, Lund, Sweden; A.H. Newman, K. Rice, J. Egan, National Inst. Diabetes, Digestive and Kidney Diseases, NIH.

LAB/BRANCH

Laboratory of Cellular Metabolism, NIH, NHLBI, Bethesda, MD

SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

National Institutes of Health, Bethesda, MD 20892

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☐ (a1) Minors

☐ (a2) Interviews

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


Isolated rat adipocytes were utilized to investigate the activation mechanisms and the role of the hormone-responsive particulate cAMP Phosphodiesterase (PDE) in insulin-dependent regulation of lipolysis. Lipolysis, cAMP-dependent protein kinase (A-kinase) and PDE were characterized under varying levels of adenylate cyclase activation and inhibition --- e.g., during activation of lipolysis by various combinations of adenosine deaminase (ADA), isoproterenol (ISO) or adenylate cyclase inhibitors, in the presence or absence of insulin.

Treatment of fat cells with either ADA or beta-agonist lead to rapid increases in A-kinase, lipolysis and particulate cAMP PDE activity. The Kact values for beta-agonist activation of both lipolysis and PDE are increased similarly as a function of increasing PIA. These data implicate A-kinase-mediated phosphorylation as a mechanism of PDE activation by lipolytic agents.

Insulin regulation of lipolysis and cAMP PDE is optimal in the presence of adenylate cyclase inhibition, suggesting a role for guanyl nucleotide-binding proteins in insulin action. In the presence of beta-agonist, insulin lowers A-kinase activity and inhibits lipolysis--provided that nanomolar PIA is present, and the A-kinase ratio is less than 0.7. In the presence of PIA (or other inhibitors), maximally-effective concentrations of insulin and ISO exert additive effects on PDE activity.

The particulate cAMP PDE from rat (and bovine) adipose tissue was solubilized with polyoxyethylene non-ionic detergents, purified to apparent homogeneity using a PDE inhibitor-affinity column and characterized in terms of sensitivity to several selective PDE inhibitors.

Project Description

Objectives:

To define mechanisms for regulation of particulate cAMP phosphodiesterase (PDE) activity in rat fat cells; to examine the role played by this enzyme in the control of lipolysis and the antilipolytic action of insulin; to investigate a role for guanyl nucleotide-binding proteins in regulation of the particulate cAMP PDE and in insulin action; to define the relationship between increases in cAMP-dependent protein kinase and changes in PDE activity mediated by lipolytic (cAMP-elevating) agents.

Methods Employed:

Adipocytes were prepared from rat epididymal fat pads of overnight-fasted animals by a modification of the method of Rodbell. Adenosine (200 nM) and glucose (2 mM) were included in all Krebs-Ringer-Hepes (KRH) buffers. Cells were diluted 5-fold in incubation buffer (4% BSA in KRH) and immediately pipetted to 25-ml flasks containing additional buffer (final volume 3.5 ml) such that the final percentage of cells (packed cell volume) was < 2%. After incubation for 20 minutes, hormones/drugs were added.

For lipolysis and cAMP-dependent protein kinase (A-kinase), incubations were terminated by homogenizing an 800-ul sample in 0.5 mM RO 20-1724/ 10 mM EDTA; glycerol content or A-kinase was assayed in the supernatant after centrifugation (30,000*g, 30 min). A-kinase activity was assayed with Kemptide as substrate with and without cAMP to determine the ratio of activation, i.e. (minus cAMP) divided by (plus cAMP); 1.0 represents maximal activation (high intracellular cAMP levels). Time-course experiments confirmed that basal A-kinase ratios were low ( <0.07) in cells prepared in the presence of adenosine, and that hormone-activated lipolysis and A-kinase ratios were being assayed under steady-state conditions.

For measurement of PDE activity, the remainder of the incubation was poured in a Dounce tube containing an equal volume of TES buffer (50 mM, pH 6.7, 5 ug/ml pepstatin, 1 ug/ml leupeptin, 0.25 M sucrose) and homogenized (8 strokes). After centrifugation (100,000*g/1 hr), the supernatants and pellets (suspended in 0.5 ml of the 1:1 TES: incubation buffer) were assayed with 0.5 uM 3H-cAMP.

Major Findings:

Lipolysis and particulate "low Km" cAMP PDE activity were compared in adipocytes under conditions of varying adenylate cyclase activation and inhibition. Adenosine deaminase (ADA) activated both lipolysis and PDE with similar concentration dependencies, presumably via removal of adenosine (Ado) and subsequent elevation of intracellular cAMP, since maximal ADA (1 U/ml) effects on both lipolysis and PDE were similarly inhibited by: (1) N6-phenylisopropyladenosine (PIA) and other ligands that inhibit adenylate cyclase (prostaglandin E1, nicotinic acid) or (2) the ADA inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine. PIA inhibition of ADA-stimulated lipolysis or PDE was reversed by the Ado antagonist 8-p-sulfo-phenyltheophylline. Particulate PDE activity was also increased by insulin and isoproterenol (ISO). With ADA, insulin did not inhibit ISO-stimulated lipolysis and the effects of insulin+ISO on PDE were equal to that of ISO alone. With ADA+PIA (or other inhibitors), insulin

inhibited ISO-stimulated lipolysis and effects of insulin+ISO on PDE were at least additive. These data indicate complex regulation of PDE in adipocytes, whereby activation of the inhibitory guanyl nucleotide-binding protein Ni modulates cAMP-activated PDE, as well as insulin-dependent PDE activity and antilipolysis.

Cyclic AMP-dependent protein kinase (A-kinase) was assayed in supernatants of adipocytes as an index of intracellular cAMP. In the presence of Ado (or PIA+ADA), the basal kinase ratio was 0.06 $\pm$ .01 (n=8), and insulin increased PDE activity by ca. 50%. ADA- or ISO (+PIA+ADA)-mediated increases in kinase and PDE activities were both maximal within 1-2 minutes and remained constant for at least 20 minutes. In the presence of ADA, the maximal A-kinase ratio was 0.32 $\pm$ 0.04 (n=6), and cAMP PDE was increased by ca. 65%. In the presence of ISO (+PIA+ADA), maximal increases in both PDE (100%) and lipolysis (400-600%) were observed at an A-kinase ratio of 0.46 $\pm$ 0.04 (n=4). The dose-response curves for ISO-mediated activation of PDE and lipolysis were superimposable and were similarly shifted to higher [ISO] in the presence of increasing [PIA] or insulin. These data implicate A-kinase-dependent phosphorylation as a mechanism by which lipolytic agents activate the particulate PDE. Insulin regulation of lipolysis and lowering of A-kinase activity was apparent only when the A-kinase ratio was less than 0.7, and at higher kinase ratios, insulin and ISO exerted additive effects on cAMP PDE activity. The additive effects on PDE activity were observed in the presence of PIA or other adenylate cyclase inhibitors, and were not associated with changes in A-kinase activity. These data indicate: spareness of A-kinase with respect to activation of lipolysis and cAMP PDE; PIA lowers A-kinase activity (via adenylate cyclase) to a level where insulin can inhibit lipolysis; and insulin activates PDE by a cAMP-independent mechanism modulated by Ni.

The putative hormone-responsive cAMP PDE was solubilized from a 100,000*g membrane fraction (prepared from rat fat pads), and purified ca. 65,000-fold to apparent homogeneity with a yield of 20% by chromatography on DEAE-Sephacel, Sephadex G-200 and affinity chromatography on aminoethyl agarose conjugated with the N-(2-isothiocyanato)ethyl derivative of the PDE inhibitor cilostamide. A 64kDa polypeptide was identified by electrophoresis in SDS; on Sephadex G-200, the apparent Mr was ca. 110,000, suggesting the native enzyme is a homodimer. The apparent Km for cAMP was 0.38 uM and Vmax 8.5 umol/min/mg; the apparent Km for cGMP was 0.28 uM and Vmax, 2.0 umol/min/mg. The purified PDE was inhibited by cGMP, cilostamide and other cardiotonic drugs, but not RO 20-1724. Based on the inhibitor studies, the hormone-sensitive "low Km" particulate cAMP PDE purified from rat adipose tissue seems to analogous to the insulin-activated form in 3T3-L1 adipocytes and the cilostamide-sensitive soluble "low Km" cAMP PDE from bovine liver, heart and human platelets. An enzyme with virtually identical properties to the rat adipose PDE was purified from bovine adipose tissue. On gel permeation, however, the apparent Mr of the bovine enzyme was 140,000, with subunits of 74 and 77 kDa identified by electrophoresis in SDS. The purified rat enzyme was a substrate for cAMP-dependent protein kinase; a single serine site was identified by peptide mapping on two-dimensional thin layer electrophoresis. The purified PDE will permit assessment of putative mechanisms (e.g., phosphorylation) of hormonal regulation.

Our data suggest that: (1) In adipocytes a specific PDE, the part-

iculate "low Km" cAMP form, is important in the metabolism of cAMP in the regulation of lipolysis and, in particular, the antilipolytic action of insulin. Analogous forms of this PDE are found in cardiac and liver tissue. (2) In addition to regulation of the synthesis of cAMP at the level of adenylate cyclase, guanyl nucleotide-binding proteins may also regulate the metabolism of cAMP via the PDE, and may be involved in insulin action. (3) Lipolytic (cAMP-elevating) agents activate the PDE as a feedback signal and/or for regulation of cAMP turnover, and the mechanism of such activation may involve cAMP-dependent phosphorylation.

Proposed Course:

(1) Characterize the effects of pertussis toxin treatment of rat adipocytes on insulin regulation of lipolysis, A-kinase and the particulate cAMP PDE; (2) characterize *in situ* pharmacology of selective PDE inhibitors in rat adipocytes with regard to lipolysis and A-kinase activation in the presence and absence of insulin; (3) determine whether *in vitro* cAMP-dependent phosphorylation of the purified PDE modulates enzyme activity; (4) using the purified PDE, appropriate tools (e.g. antibodies) will be developed to study the phosphorylation state and subcellular localization of this enzyme in rat adipocytes, the appearance of this enzyme during the differentiation of 3T3-L1 fibroblasts into adipocytes, the relationship between analogous PDE types in adipose, cardiac and hepatic tissues, and to assess the importance of the PDE in pathological states, i.e. diabetes.

Publications

Degerman, E., Belfrage, P., Hauck Newman, A., Rice, K.C., and Manganiello, V. C. (1987) Purification of the putative of the hormone-sensitive cyclic AMP phosphodiesterase from rat adipose tissue using a derivative of cilostamide as a novel affinity ligand. *J. Biol. Chem. 262:* 5797-5807.

Elks, M. L., Jackson, M. J., Manganiello, V. C. and Vaughan, M. (1987) Effect of N6-(L-2-phenylisopropyl)adenosine and insulin on cAMP metabolism in 3T3-L1 adipocytes. *Amer. J. Physiol. 252 (Cell Physiol. 21):* C342-C348.

Manganiello, V. C., Degerman, E., and Elks, M. L. (1987) Use of selective phosphodiesterase inhibitors in intact cells. *Methods in Enzymology* (in press).

Manganiello, V. C. and Elks, M. L. (1986) Regulation of particulate cAMP phosphodiesterase activity in 3T3-L1 adipocytes: the role of the particulate phosphodiesterase in the antilipolytic action of insulin. *Mechanisms of Insulin Action.* Belfrage, P., Donner, J and Stralfors, P., eds. Elsevier Science Publishers, pp. 147-166.

Manganiello, V. C., Smith, C. J., Degerman, E., Hauck Newman, A., Rice, K.C. and Belfrage, P. (1987) Hormonal regulation of particulate cAMP phosphodiesterase in adipocytes. *J. Cyclic Nuc. and Prot. Phos. Res.* (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00638-05 CM

PERIOD COVERED
October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Genes for GTP-binding Proteins

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

| | | | |
|---|---|---|---|
| PI: | S. Russ Price, Ph.D. | Guest Researcher | CM, NHLBI |
| Others: | Krisa Van Meurs, M.D. | Guest Researcher | CM, NHLBI |
| | Inez Serventi, Ph.D. | Staff Fellow | CM, NHLBI |
| | James Murtagh, M.D. | Md. Staff Fellow | CM, NHLBI |
| | Eleanor Bruckwick | Chemist | CM, NHLBI |
| | C. William Angus, Ph.D. | Staff Fellow | CM, NHLBI |
| | Joel Moss, M.D., Ph.D. | Head, Sec. Mol. Mech. | CM, NHLBI |
| | Martha Vaughan, M.D. | Chief | CM, NHLBI |

COOPERATING UNITS (if any)
Dr. H-F. Kung, Chief and Dr. Sukadev Lavu, Senior Staff Fellow, Laboratory of Biochemical Physiology, NCI, FCRF, Frederick, MD 21701

LAB/BRANCH
Laboratory of Cellular Metabolism, NIH, NHLBI, Bethesda, MD

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
National Institutes of Health, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 5.0 | 4.0 | 1.0 |

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

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

Guanyl nucleotide-binding proteins (GNPs) are involved in the regulation of receptor mediated transmembrane signal transduction. The retinal photon receptor, rhodopsin, is linked to a cGMP phosphodiesterase by the GNP, transducin (Gt). Stimulatory and inhibitory receptors of the adenylate cyclase system are coupled to the catalytic subunit through two GNPs, Gs and Gi, respectively. A fourth GNP, Go, functionally interacts with muscarinic receptors and rhodopsin; however, its physiological function is unclear. All of these GNPs exhibit functional similarities and structurally are composed of α, β, and γ subunits.

A number of cDNA clones have been isolated from a bovine retinal library and one (λGo9) has been sequenced in its entirety with the deduced amino acid sequence corresponding to Goα. Other clones appear to be related to but not necessarily identical to λGo9.

Additionally, a bovine genomic DNA library was screened using the Goα cDNA, λGo9, as a probe. A number of clones of varying intensities of hybridization have been isolated and purified. Restriction analysis and hybridization with various probes corresponding to different regions of the cDNA have revealed that at least three related but different clones have been identified.



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

Project No. Z01 HL 00638-05 CM

Project Description:

Objectives: To clone and characterize cDNAs which code for the subunits of guanyl nucleotide-binding proteins (GNPs) and to utilize these cDNAs to study the genetic organization, expression and function of these proteins. Two GNPs ($G_s$ and $G_i$) are involved in coupling of stimulatory and inhibitory receptors with the adenylate cyclase system. In retina, two transducins ($G_{t1}$ and $G_{t2}$ from rod and cone cells, respectively) link the photon receptor rhodopsin to a cGMP phosphodiesterase. Another GNP, $G_o$, is found predominately in brain and interacts functionally with muscarinic receptors and rhodopsin.

A characteristic shared by all GNPs is their heterotrimeric structure consisting of α, β, and γ subunits. The α subunits vary in size from 39 kDa to 45 kDa. They exhibit a GTP-binding site and GTPase enzymatic activity. The β subunits (35-36 kDa) are required for maximal expression of GTPase activity by the α subunit. The γ subunits (6-8 kDa) are tightly bound to the β subunits and have an undetermined function. cDNAs sequences for the subunits of $G_s$, $G_i$, and $G_t$ have been published. Deduced amino acid sequences indicate regions of extensive similarity among GNPs. These data are consistent with previously observed immunological characteristics, structural data, ADP-ribosylation by choleragen and pertussis toxins, and functional similarities in reconstituted systems. We have utilized a cDNA clone for $G_{o\alpha}$ (λGo9), isolated and sequenced in this laboratory, as well as oligonucleotide probes corresponding to specific regions of GNPs to screen cDNA and genomic libraries for genes for $G_{o\alpha}$ and related proteins. Isolation and sequence analysis of GNPs will provide information pertinent to the structure, function and expression of these regulatory proteins.

Methods Employed: Isolation of cDNAs related to $G_{o\alpha}$. A bovine retinal cDNA library in λgt10, kindly provided by Dr. Jeremy Nathans was screened by standard plaque hybridization techniques with $^{32}$P-labeled $G_{o\alpha}$ cDNA and $^{32}$P-5'-end labeled oligonucleotide probes corresponding to nucleotide sequences that are either unique to $G_{o\alpha}$ or homologous among GNPs.

Restriction enzyme analysis. cDNAs, identified by hybridization with $^{32}$P-labeled probes and plaque purified, were digested with various restriction endonucleases and the fragments separated by electrophoresis on a 0.8% agarose gel. Following transfer of the DNA fragments to nitrocellulose, the filters were hybridized to $^{32}$P-labeled probes to identify fragments containing related GNP nucleotide sequences.

315

Isolation of a gene encoding $G_{o\alpha}$. A bovine genomic DNA library in Charon 28, kindly provided by Dr. Fritz Rottman was screened using standard protocols with $^{32}P$-labelled $G_{o\alpha}$ cDNA, which had been prepared by the multiprimer extension method. Positive clones were plaque purified and DNA was isolated using the Lambda-Sorb method. Purified DNA was further analyzed with restriction endonucleases to find overlapping clones and hybridization analysis was performed with $^{32}P$-labelled restriction fragments of the $G_{o\alpha}$ cDNA to map regions of the gene corresponding to appropriate regions of the cDNA. Restriction fragments of genomic inserts were purified from the Charon 28 vector by elution from a 0.8% agarose gel. Purified fragments were subcloned into a variety of vectors (pUC 19, pBR 322 and Bluescript M13) for sequencing.

Major Findings: We previously reported isolation of a $G_{o\alpha}$ clone (λGo9) from a bovine retinal λgt10 cDNA library. λGo9, was subcloned into M13mp18 and M13mp19 for Sanger dideoxy sequencing and into pSVL for Maxam and Gilbert sequencing, which was completed this year. λGo9 was found to have an open reading frame of 354 amino acids with a calculated molecular mass of 39.9 kDa. Comparison of the nucleotide sequence of λGo9 with a published partial cDNA clone for rat $G_{o\alpha}$ reveals 92% identity in coding and 3' untranslated regions. The deduced amino acid sequences of bovine $G_{o\alpha}$ and other bovine GNPs were also compared. $G_{o\alpha}$ is most similar to $G_{i\alpha}$ with 73% identity and 82% homology when conservative substitutions are included. The transducins $G_{t1}$ and $G_{t2}$ have 60% and 61% identity and 76% and 78% homology with $G_{o\alpha}$ respectively. $G_{s\alpha}$ is most different with only 34% identity and 50% homology with $G_{o\alpha}$. It has also been noted that there are marked similarities of sequence in regions of the GNPs, elongation factors, and ras p21 gene products that are believed to be involved in guanine nucleotide-binding and GTP hydrolysis.

The cDNA λGo9 encoding the complete amino acid sequence of $G_{o\alpha}$ was used to screen the retinal library for related cDNAs. Of 400,000 plaques screened with this cDNA, 30 positives were identified and plaque purified. Restriction analysis of the cDNA clones that a number of distinct clones may have been isolated.

A Charon 28 bovine genomic DNA library was screened using $^{32}P$-labelled restriction fragments of the $G_{o\alpha}$ cDNA. Twenty-four positive clones of varying hybridization intensities were identified. Ten of the most strongly hybridizing clones were characterized further, five of which were plaque purified. Restriction digest and hybridization patterns revealed that three of the five clones were identical and hybridized to a different region of the cDNA than did the remaining two clones. Although these two clones hybridized to similar regions of the cDNA and

bore some resemblance in restriction digest pattern, they appeared to be different from each other. Many of the inserts have been subcloned in preparation for more detailed restriction analysis and sequencing.

Proposed Course: 1) To sequence the remaining isolated cDNA clones to obtain the deduced amino acid sequences and thus their identity.
2) To sequence the isolated genomic clones to obtain information regarding the intron/exon structure of the gene as well as the presence of regulatory elements in the noncoding regions of the gene.
3) To construct mutant GNPs for study of structure-function relationships of GNPs.

Publications:

Angus, C.W., Van Meurs, K.P., Tsai, S-C., Adamik, R., Miedel, M.C., Pan, Y-C.E., Kung, H-F., Moss, J., and Vaughan, M.: Identification of the probable site of choleragen-catalyzed ADP-ribosylation in a $G_{o\alpha}$-like protein based on cDNA sequence. Proc. Natl. Acad. Sci. USA 83: 5813-5816, 1986.

McEnery, M.W., Angus, C.W., and Moss, J.: Affinity chromatographic procedure for the quantitative recovery of DNA fragments from agarose gels. Anal. Biochem. 156: 72-75, 1986.

Van Meurs, K.P., Angus, C.W., Lavu, S., Kung, H-F., Czarnecki, S.K., Moss, J., and Vaughan, M.: Deduced amino acid sequence of bovine retinal $G_{o\alpha}$: Similarities to other guanine nucleotide-binding proteins. Proc. Natl. Acad. Sci. USA 84:3101-3111, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00639-04 CM

PERIOD COVERED
October 1, 1986 through September 30, 1987

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 Bio. Physiology | CM, NHLBI |
| Others: | Seiko Murashima, M.D., Ph.D. | Visiting Fellow | CM, NHLBI |

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cellular Metabolism, NIH, NHLBI, Bethesda, MD

SECTION
Biochemical Physiology

INSTITUTE AND LOCATION
National Institutes of Health, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.8 | 0.3 | 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.)
Bovine ROS cGMP PDE and bovine brain particulate and soluble cGMP-stimulated PDEs were purified. Treatment of these PDEs with trypsin and chymotrypsin suggest that these PDEs exhibit differing susceptibilities to proteolytic degradation and liberate different major peptides during proteolytic digestion.



PHS 6040 (Rev. 1/84) GPO 914-913

Project Description

Objectives: To characterize regulatory and structural properties of ROS cGMP PDE; a comparison of some of the characteristics of ROS cGMP PDE and cGMP-stimulated PDE.

Methods: Hypotonic extracts of ROS were applied to aminopentyl agarose. The column was washed with HEPES buffer containing 75 and 150 mM NaCl. ROS cGMP PDE was eluted with 300 mM NaCl. After concentration, ROS cGMP PDE was further purified by FPLC size exclusion chromatography (Superose 6). cGMP-stimulated PDEs were purified by affinity chromatography on cGMP-epoxyagarose. Purity was assessed via SDS-PAGE.

Proteolysis was carried out in 50 mM HEPES buffer (pH 7.5) containing 50 mM NaCl. PDE was incubated with trypsin or chymotrypsin (<5 µg) at 30°C. The reaction was terminated by addition of 50 mg soybean trypsin inhibitor. Some samples were precipitated with TCA and analyzed via SDS PAGE and Western immunoblots.

Major Findings: A rapid method for purification of ROS PDE was developed. Approximately 80-90% of the original hypotonic extract does not adsorb to amino pentyl agarose columns, whereas virtually all of the ROS cAMP PDE binds and can, after washing the columns with 75 and 150 mM NaCl, be eluted in good yield with HEPES buffer containing 300 mM NaCl. After concentration, highly purified PDE can be prepared by FPLC size exclusion chromatography on Superose 6. Particulate and soluble cGMP-stimulated PDEs, with apparently different monomeric subunit $M_r$ values, were isolated from bovine brain.

Brief incubation of the soluble cGMP stimulated PDE with trypsin resulted in rapid degradation and loss of immunoreactive material with $M_r$ ~ 102 kDa; no smaller immunoreactive peptides were observed. The particulate cGMP-stimulated PDE which exhibits a slightly larger subunit size was more stable to trypsin digestion, with formation of several major smaller peptides ($M_r$ ~ 50 kDa to 60 kDa). The ROS PDE was also relatively stable to trypsin and chymotrypsin digestion. During treatment with trypsin, a number of peptides were formed with $M_r$ 50 to 90 kDa. During treatment with chymotrypsin, however, a more discrete pattern was observed with 4 major immunoreactive peptides in the ~ 50 to 90 $M_r$ range. The major peptides generated from the different PDEs exhibit different $M_r$ values.

We wish to make further comparisons between the three PDEs with respect to effects of proteases and photoaffinity labelling with cGMP.

Significance to Biomedical Research and the Program of the Institute: The ROS cGMP phosphodiesterase, which plays a major role in the process of visual excitation, is controlled through a

receptor/GTP-binding protein system analogous to that of the hormone-sensitive adenylate cyclase. The ROS PDE and cGMP-stimulated PDE exhibit different catalytic properties and binding characteristics for the same substrate, i.e., cGMP. Understanding the structure and regulatory properties of these enzymes will be important in understanding mechanisms of cGMP action as well as the molecular basis for diversity among various PDEs tissue.

Proposed Course: Additional characterization of the regulatory properties of this enzyme and comparison with other PDE's especially the cGMP stimulated and hormone-sensitive "low $K_m$" particulate cAMP PDE.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00643-01 CM

PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Heterologous Expression of Guanyl Nucleotide Binding Proteins


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

| | | | |
|---|---|---|---|
| PI: | C. William Angus, Ph.D. | Staff Fellow | CM, NHLBI |
| Others: | Suzanne K. Czarnecki, Ph.D. | Staff Fellow | CM, NHLBI |
| | Joel Avigan, Ph.D. | Res. Chemist | CM, NHLBI |
| | Joel Moss, M.D., Ph.D. | Head, Section on Molecular Mechanisms | CM, NHLBI |


COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular Metabolism, NIH, NHLBI, Bethesda, MD

SECTION

Molecular Mechanisms

INSTITUTE AND LOCATION

National Institutes of Health, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.9 | 2.9 | 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.)


Go is a member of a family of guanyl nucleotide binding proteins (GNPs) which are involved in the regulation of receptor-associated phenomena. Go has been shown to interact functionally with the photo-receptor rhodopsin and with muscarinic receptors. As has been observed with all other GNPs, Go exists as a heterotrimer of α, β and γ subunits.

A cDNA clone, containing the complete protein coding sequence for the α subunit of bovine retina Go (Goα) has been isolated and sequenced. This clone has been utilized for expression in both prokaryotic and eukaryotic hosts. The cDNA, starting with the ATG initiation codon and comprising the entire coding region and approximately 200 bases of the 3' untranslated region was ligated into the EcoR1 site of pRC-23, an E. coli expression vector. Upon induction, Goα is produced at a level approximating 1-2% of the total cellular protein. As demonstrated by western analysis, this recombinant protein (rGoα) is immunoreactive with polyclonal antisera. rGoα also serves as a substrate for ADP-ribosylation by pertussis toxin. Stimulation of pertussis toxin catalyzed ADP-ribosylation of rGoα is observed in the presence of the β and γ subunits of transducin.

Gene transfer experiments have been carried out in which the Goα cDNA was transfected into COS cells, utilizing a vector with an SV40 promoter. Cytoplasmic dot-blot assays demonstrated that transcription occurred within 12 hours of uptake and continued for up to 80 hours.

Project No. Z01 HL 00643-01 CM

Project Description

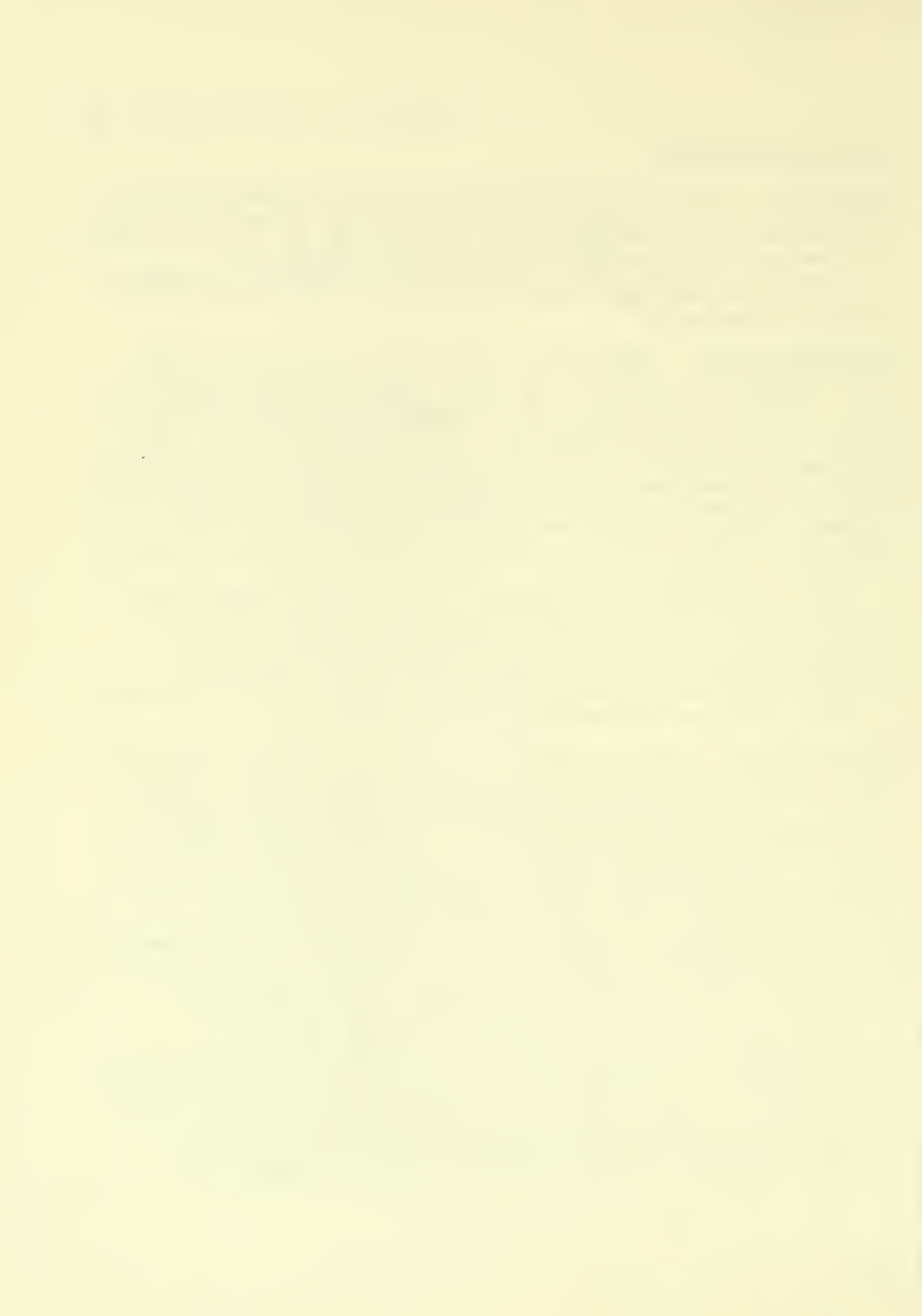
Objectives: To express the α-subunit of $G_o$ in prokaryotic and eukaryotic systems. Expression of the protein in mammalian cells will enable further studies to be performed on the function of $G_o$ in these cells. Expression in E. Coli will facilitate purification of this protein and will also allow for production of $G_{o\alpha}$ with defined mutations, which can be used in structure-function experiments.

Methods Employed: Expression of $G_{o\alpha}$ in E. Coli was performed utilizing pRC-23, a gift of Dr. Robert Cowl, Hoffman-La Roche, Inc. An Nco1-EcoRI digest of the $G_{o\alpha}$ cDNA was blunt-ended with Klenow polymerase and ligated into the blunt-ended EcoRI site of pRC-23, a vector which contains the λpL promoter region. The resultant recombinant plasmid was designated pRC-4. Transfection into E. Coli was done utilizing strain pRI [pRK 248 cIts], which contains a plasmid encoding the temperature sensitive lamda repressor λcIAt2. Production of $rG_{o\alpha}$ synthesis was performed by raising the temperature of a logarithmically growing culture to 42°C for 2 hours.

The plasmid pSVL (Pharmacia) was used for transfection of COS cells. A 1.9 Kb cDNA clone for $G_{o\alpha}$, containing the entire coding sequence was blunt-ended into the SmaI site of pSVL. Transfection into COS cells was performed by the calcium phosphate method. RNA was isolated by NP-40 disruption of monolayers followed by formaldehyde treatment. $^{32}P$-radiolabeled $G_{o\alpha}$ cDNA probe was synthesized by nick-translation.

Major Findings: Western analysis of induced bacterial cultures containing pRC-4 indicated that a protein was produced which was immunoreactive with polyclonal antisera raised against bovine brain $G_{o\alpha}$. As determined by SDS-PAGE, the molecular weight of the recombinant protein (39 kDa) was indistinguishable from that reported for bovine brain $G_{o\alpha}$. Densitometric analysis revealed that $rG_{o\alpha}$ was produced a level representing 1-2% of the total cellular protein. As isolated from E. Coli by lysozyme disruption, $rG_{o\alpha}$ appears as a complex of approximately 160 kDa upon chromatography on Sephacyl S-200 (Pharmacia). Treatment of the bacterial lysate with a number of non-ionic (Lubrol, Tween-20) or ionic (cholate, CHAPS) detergents did not alter the apparent molecular weight. Disruption of the bacteria by sonication also failed to reduce the size of the pellet.

After the crude bacterial lysate was subjected to chromatography on Sephacyl S-200 the $rG_{o\alpha}$ was found to serve as a substrate for ADP-ribosylation by pertussis-toxin utilizing $^{32}P$-NAD as donor. Stimulation of this toxin catalyzed modification was observed when the β and γ subunits of bovine retina transducin another GNP, were added to the toxin-catalyzed reaction. This result indicated that $rG_{o\alpha}$ was capable of



functionally interacting with $\beta$ and $\gamma$ subunits of the heterotrimer. Presently, oligonucleotide site-directed mutagenesis experiments are being conducted to alter sites in $rG_{o\alpha}$. Purification of $rG_{o\alpha}$ is also proceeding.

Gene transfer experiments are in progress to analyze the expression of bovine retinal $G_{o\alpha}$ in mammalian cells. A 1.9 Kb full length $G_{o\alpha}$ cDNA was blunt-end ligated into the eukaryotic shuttle vector pSVL (Pharmacia) and transfected into exponentially growing COS cells by the calcium phosphate precipitation method. In this system, transient RNA synthesis is directed by the plasmids SV40 transcriptional elements. Transcription occurs within 12 h of uptake and continue for up to 80 h following transfection as determined by cytoplasmic dot blot assay using a nick-translated cDNA probe. Mock infected cells do not product $G_{o\alpha}$ mRNA.

Publications:

Rizzo, W. B., Phillips, M. W., Dammann, A. L., Leshner, R. T., Jennings S. S., Avigan, J., and Proud, V. K.: Adrenoleukodystrophy: dietary oleic acid lowers hexacosanoate levels. Ann. Neurol. 21: 232-239, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00644-01 CM

PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Guanyl Nucleotide-Sensitive α1-adrenergic Receptor

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


| | | | |
|---|---|---|---|
| PI: | Stuart I. Harris, Ph.D., M.D. | Med. Staff Fellow | CM, NHLBI |
| Others: | Inez M. Serventi, Ph.D. | Staff Fellow | CM, NHLBI |
| | Joel Moss, M.D., Ph.D. | Head, Section on Molecular Mechanisms | CM, NHLBI |


COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular Metabolism, NIH, NHLBI, Bethesda, MD

SECTION

Molecular Mechanisms

INSTITUTE AND LOCATION

National Institutes of Health, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.3 | 1.3 | 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.)


Studies with isolated plasma membranes indicate that agonist binding to the α1-adrenergic receptor may be regulated by GTP and its nonhydrolyzable analogues. To further study α1-receptor:effector interactions, a soluble hormone-receptor complex was prepared by incubating rat liver membranes with norepinehrine followed by detergent solubilization with digitonin. The addition of guanyl nucleotides to the solubilized receptors facilitated the release of tightly bound norepinephrine. Binding of the α1-adrenergic receptor antagonist, [3H]-prazosin, to the soluble preparation was utilized as a gauge of guanyl nucleotide-induced release of receptor-bound agonist. The following potency series was obtained with regard to the ability of guanyl nucleotides to facilitate [3H]-prazosin binding to the solubilized preparation: guanosine 5'-0-(3-thiotriphosphate)>guanylyl-β,γ,imidodiphosphate>guanosine triphosphate>>>adenylyl-β,γ,imidodiphosphate. Competitive binding studies indicated that the receptor had a higher affinity for prazosin than for either the non-selective alpha antagonist phentolamine, or the α2-selective antagonist, yohimbine, thereby confirming the receptor's identity as the α1-adrenergic subtype. Photo-affinity labeling studies with the prazosin analog [125I]-aryl azidoprazosin indicated a molecular weight of ~ 90,000 for the guanyl nucleotide-sensitive α1-adrenergic receptor.





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

Utilizing the photoaffinity prazosin analog, [$^{125}I$]-aryl azidoprazosin, the guanyl nucleotide-sensitive $\alpha_1$-adrenergic receptor was identified under reducing conditions as a protein with $M_r \sim 90,000$ on sodium dodecyl sulfate - polyacrylamide gel electrophoresis.

Significance to Biomedical Research and the Program of the Institute: In addition to the $\alpha_1$-adrenergic receptor, the vasopressin and angiotensin receptors are felt to modulate intracellular calcium levels through a guanyl nucleotide-dependent signal transduction mechanism. All of these hormones cause peripheral vasoconstriction and thus understanding their mechanism of action is central to understanding blood pressure regulation and hypertension. Our demonstration of guanyl nucleotide-sensitive binding in a soluble preparation should permit further biochemical manipulations aimed at understanding the coupling between receptor and guanyl nucleotide-binding protein.

Proposed Course: 1) Further characterize the $\alpha_1$-adrenergic/ receptor guanyl nucleotide binding protein interaction and distinguish its behavior from that of other adrenergic receptor subtypes found in the liver. 2) Pursue identification of the guanyl nucleotide-binding protein coupled to the hepatic $\alpha_1$-adrenergic receptor.

Publications: None.

ANNUAL REPORT OF THE
LABORATORY OF CHEMICAL PHARMACOLOGY
National Heart, Lung, and Blood Institute
October 1, 1986 to September 30, 1987

During the past several years, this Laboratory has been studying possible mechanisms by which drugs, other foreign compounds and their metabolites may evoke various kinds of toxicities. Most of the Laboratory has focused on various aspects of the mechanisms of immune reactions. One section has directed attention on mechanisms by which chemically reactive metabolites react with cellular components to form putative antigens which may be important in mechanisms of immune mediated mechanisms of cellular toxicity. Another section has directed attention on the mechanisms by which antigens evoke the release of histamine and other substances from granules in mast cells. In addition the Laboratory has continued its efforts in identifying isozymes of cytochrome P-450 that catalyze the metabolism of foreign compounds and in discovering the factors that govern the formation of different metabolites by individual enzymes. The Laboratory is continuing to develop pharmacokinetic tools for elucidating the kinetics of short-lived metabolites that play important roles in drug-induced toxicities.

## Mechanisms of Toxicity

Halothane, enflurane, and isoflurane - There is considerable evidence that the fulminant type of hepatitis caused by halothane in humans may be due to an immune reaction. Sera of patients with halothane-induced hepatotoxicity contain antibodies that in the presence of human lymphocytes kill hepatocytes from rabbits treated with halothane but not those from untreated rabbits. The various antibodies in the sera react to different extents with several hepatic microsomal proteins (100 kDa, 76 kDa, 58 kDa, 57 kDa and 54 kDa) from rabbits treated with halothane or from humans exposed to halothane. The antibody reactions, however, are much less extensive with the hepatic proteins from untreated rabbits and humans. The antibodies are not present in sera of patients not manifesting hepatitis but receiving halothane nor are they present in sera of patients manifesting other types of hepatic disease. During the past year we have found that the antibodies react with hepatic microsomal proteins from rats treated with halothane and have thus been able to use rats as an animal model to study other properties of the epitopes recognized by the antibodies. Thus far we have found that all of the epitopes contain the trifluoracetyl group of halothane, but must also include portions of the proteins adjacent to the amino acids that bind trifluoroacetyl groups. Studies *in vitro* have revealed that the trifuoroacetyl group is formed during the oxidative rather than the reductive metabolism of halothane.

In previous years, we developed an antibody against trifluoroacetyl lysine. This year we have found that this antibody recognizes not only proteins containing irreversibly bound metabolites of halothane but also irreversibly bound metabolites formed from enflurane and isoflurane administered to rats. The relative rates of formation of the epitopes are halothane > enflurane > isoflurane. Immunoblotting techniques have revealed that the reactive metabolites

of enflurane and halothane react with the same proteins. The binding of the reactive metabolite of isoflurane, however, was too small to analyze. Antibodies in the sera of patients also reacted with the epitopes formed during the metabolism of isoflurane, which lends support for the belief that patients that are sensitive to halothane would also be sensitive to enflurane and perhaps to isoflurane.

As reported last year the antibody against trifluoroacetyl lysine reacts with a 59 kDa protein in liver microsomes from rats treated with halothane. This protein, which has been purified by adsorption onto an affinity gel column containing covalently bound rabbit anti-trifluoroacetyl lysine IgG and elution with trifluoroacetyl lysine, was found to be an isozyme of cytochrome P-450. An antibody against the purified protein (anti-59 kDa) was prepared and was found to react with a 59 kDa protein in microsomes of kidney, lung, adrenals, testes, ovaries and adipocytes as well as liver of untreated rats and with a 59 kDa protein in human liver. Even though the purified protein reacts with anti-trifluoroacetyl lysine IgG, it does not react with any of the sera from patients with halothane-induced hepatitis. It thus is not the 54 k Da, 57 kDa or the 58 kDa proteins that react with these sera.

The anti-59 kDa IgG reacts with a preparation, isolated from rabbit liver by Dr. Kupfer, that hydroxylates the $\omega$-position of prostaglandins. Since the antibody does not inhibit the reaction, however, it has not yet been unequivocally established whether it reacts with the $\omega$-hydroxylase or with a protein contaminant that comigrates with the $\omega$-hydroxylases.

Mechanisms of heme destruction - It has been established that many substances including allylic substances that cause porphyria and carbon tetrachloride inactivate cytochrome P-450 by causing the destruction of the heme in the cytochrome P-450, probably through a free radical mechanism. During the past year, we have found that during the inactivation of purified [$^{3}$H-heme]-labeled cytochrome P-450 54 kDa by $^{14}C\ Cl_4$ in a reconstituted system, the Soret band of the heme is lost and the $^{14}C$ becomes covalently bound to the inactivated cytochrome P-450 but not to the cytochrome P-450 reductase. Moreover, subjection of the inactivated cytochrome P-450 to proteolysis by protease K gave two products containing $^{3}H$; the $^{14}C/^{3}H$ molar ratio of one of the products was 4-5/1 whereas that of the other was 9-10/1. The high incorporation of $^{14}C$ into the products suggests that several molecules of $CCl_4$ are metabolized before the enzyme becomes inactivated.

Gamma radiolysis of myoglobin in the presence of $CCl_4$ also leads to covalent binding of the heme with protein but without destruction of the Soret band, possibly because the reaction of the $CCl_3$ radical initiates covalent binding of the heme to protein without loss of the heme ring system.

Cardiotoxicity caused by anthracyclines - The usefullness of anthracyclines in the treatment of various cancers has been limited largely because these drugs cause cardiotoxicity. During the past several years we have found that these drugs kill cardiomyocytes in culture apparently by mechanisms that lead to

the early loss of ATP and GSH followed by leakage of adenine nucleotides and lactate dehydrogenase. The effects depend not only on the concentration of the anthracyclines but also on the time of incubation. Low concentrations ($< 1$ µM) still cause loss of ATP and GSH, but the loss is delayed compared with the losses obtained with higher concentrations. If the usefullness of this group of drugs is to be improved, then it will be necessary to demonstrate that the mechanism of cardiotoxicity differs from the mechanism of their therapeutic effects. That this may be possible is illustrated by studies during the past year showing that various anthracyclines inhibit the growth of L 1210 leukemia at lower concentrations than those which cause loss of ATP and GSH in cardiomyocytes and that the relative potencies of the analogs in causing the two effects differ markedly with the analogue used.

Mechanism of hepatocytes toxicity caused by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) - This designer drug causes a Parkinson-like syndrome in humans and primates. It is now believed that the drug evokes its toxicity through the conversion of MPTP by monoamine oxidase B to $MPP^+$ (1-methyl-4-phenyl pyridinium ion), which undergoes redox cycling with the formation of superoxide and hydrogen peroxide. Deprenyl, which inhibits monoamine oxidase B, effectively prevents the depletion of dopamine in the striatum caused by MPTP in rats. During the past year, we have studied the effectiveness of several analogues of diprenyl in preventing hepatocyte death caused by MPTP in culture and have found that several analogues are more potent than deprenyl. The apparent $IC_{50}$ values in assay systems in which rat hepatocytes were preincubated with the inhibitors for 2 hr were: Deprenyl, 11 µM; T2650, 3 µM; U 1424, 2 µM and J 508, 0.5 µM. Preliminary studies indicate that these inhibitors are also effective in preventing the loss of dopamine from the striatum of rat brain caused by MPTP.

Mechanism of Mast Cell Activation and Degranulation

Although the mast cell and its related cultured-cell lines (e.g. the RBL-2H3 cell), are in themselves useful experimental models for studies of the mechanism of $Ca^{2+}$-dependent secretion, our objective is to study the mechanism as a basis for developing appropriate therapies for the suppression of secretion. The abundance of mast cells in blood vessels, heart and airways makes these sites especially vulerable to the actions of histamine and other potent inflammatory mediators that are released from these cells in response to IgE-directed antigens. Our studies over the past 5 years provide convincing evidence that antigen-induced breakdown of membrane inositol phospholipids can generate the necessary signals for secretion. This past year we have been able to define these stimulatory events in molecular terms. Although some of these events appear to be identical to those previously described for other types of cells, some of our findings are novel and have revealed unexpected aspects of the phosphoinositide signal transduction mechanism.

We have explored many aspects of the system ranging from the aggregation of receptors for IgE on the plasma membrane to the phosphorylation of proteins that regulate contractile elements in the cell. We showed previously that the hydrolysis of the inositol phospholipids (to form inositol phosphates and diacylglycerol) was governed by the number of receptors aggregated. In addition,

a variety of experimental maneuvers that perturbed this response resulted in corresponding increases or decreases in the intensity of the calcium signal (i.e. the rise in levels of cytosol $Ca^{2+}$) and in the extent of the secretory response. Moreover, displacement of the IgE cross-linking antigen with an excess of monovalent hapten abrogated the stimulatory responses (i.e. hydrolysis of the phospholipids and calcium signal) and secretion.

As indicated from our recent studies, the stimulatory responses are also markedly dependent on the efficacy of the cross-linking agent in inducing aggregation of IgE receptors. For example, in the absence of external $Ca^{2+}$, the responses varied from nothing (with aggregated ovalbumin) to a transient burst in production of inositol phosphates (with $DNP_{24}BSA$:24 molecules of dinitrophenol conjugated to one molecule of bovine serum albumin). Small calcium signals were generated only when the cross-linking agent caused the production of inositol phosphates. In the presence of external $Ca^{2+}$, responses to all agents were amplified in proportion to the concentration of external $Ca^{2+}$; at concentrations equal to or greater than 0.1 mM secretion of histamine occurred.

A significant technical achievement was the finding that treatment with the toxin streptolysin O resulted in complete permeabilization of RBL-2H3 cells without impairment of the antigen induced hydrolysis of inositol phospholipids or secretion. This has enabled us to alter the "internal" environment of the cell and thereby to study the factors that govern the production of messenger molecules at the level of the membrane as well as the intracellular processes that regulate secretion. The studies revealed that the phosphoinositide cycle was more complex than was envisaged previously. Antigen stimulation of RBL-2H3 cells resulted in the simultaneous hydrolysis of phosphatidylinositol-4-monophosphate (to produce inositol 1,4-bisphosphate) and phosphatidylinositol-4,5-bisphosphate (to produce inositol 1,4,5-trisphosphate). Conversion of the inositol 1,4,5-trisphosphate to another putative messenger inositol 1,3,4,5-tetrakisphosphate, was shown to be a reversible process. As outlined in the individual project reports, the sequential dephosphorylation of the two inositol polyphosphates proceeded through several pathways. Possibly the most seminal observation was that the rates of production of inositol phosphates and of the conversion of the trisphosphate to the tetrakisphosphate were increased by increasing the concentration of free $Ca^{2+}$ from <5 to 1000 nM. The data thus supported our earlier notion that small stimulatory responses that were evoked in the absence of external $Ca^{2+}$ could be markedly enhanced by increases in cytosol $Ca^{2+}$ and that an early stimulatory event was the mobilization of intracellular $Ca^{2+}$ by inositol 1,4,5-trisphosphate which was subsequently reinforced by influx of external $Ca^{2+}$ ions. Although the mechanisms of $Ca^{2+}$-influx in RBL-2H3 cells has not been elucidated, one candidate from studies with the patch clamp technique (with J. Barker, LNP, NINCDS) is a combination of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate both of which activate the same ion-channel activities in the plasma membrane as antigen.

Neither the calcium signal nor the activation of protein kinase C by diacylglycerol alone are sufficient stimuli for secretion. Both processes are required. Our recent studies with the permeabilized cells indicate the possible mechanism of these potentiating interactions. Secretion is induced by $Ca^{2+}$

alone at concentrations above 1 μM in a concentration-dependent fashion, but when hydrolysis of inositol phospholipids is induced either by antigen or by stimulation of GTP-binding proteins (with GTP qS) the minimal concentration of $Ca^{2+}$ that is required to support secretion is lowered to about 0.1 μM. We presume that activation of protein kinase C by diacylglycerol sensitizes the cell to the action of $Ca^{2+}$. This point will be investigated in future studies by exhaustively dialyzing the cell to remove protein kinase C and by performing reconstitution studies with the purified enzyme.

Studies performed in collaboration with Dr. R. Adelstein support the notion that both $Ca^{2+}$-dependent and protein kinase C-dependent processes are brought into play during antigen stimulation. Two distinct sites on the light chain of myosin are phosphorylated. On one site phosphorylation is catalyzed by the $Ca^{2+}$, calmodulin-dependent myosin kinase. This site is phosphorylated transiently: phosphorylation occurs within seconds of addition of antigen but dephosphorylation also occurs rapidly. Phosphorylation of the second site is catalyzed by protein kinase C. Phosphorylation of this site correlates over time with the rate of secretion.

We have now characterized individual variants of the RBL-2H3 cell that have no calcium signal, lack protein kinase C, or have a defective coupling of receptors to phospholipase C but retain a competant mechanism for secretion: when challenged with $Ca^{2+}$-specific ionophores and exogenous activators of protein kinase C they show a normal secretory response. Studies with these variants improve our prospects of elucidating the roles of the various processes described above.

## Biochemistry and Kinetics of Drug Metabolism

Metabolism of foreign compounds by isozymes of cytochrome P-450 synthesized from cDNA's transfected into cells - Last year, we reported that Gonzalez (NCI) had used one of our antibody preparations to identify cDNA clones that code for cytochrome P-450a and has transfected the cDNA into both yeast and COS cells. Since cytochrome P-450a catalyzed the conversion of testosterone to both 7α-testosterone and 17β-hydroxy-4,6-androstadiene-3-one (6-delta t), we plan to use the transfected preparations for studying the properties of the active site of the enzyme and the physico chemical properties of various substrates that determine the substrate specificity and product specificity of the isozyme.

Our initial efforts will be focused on determing whether the formation of 6-delta t occurs by a double hydrogen abstraction mechanism. We have previously proposed this mechanism for the formation of 6-delta t by 6β-hydroxylases in rat liver microsomes, based on evidence indicating that 6-delta t did not contain the 7α-hydrogen of testosterone. The apparent lack of an isotope effect, however, has worried us. During the past year, however, we began to realize that a very strong [7α-$^{3}$H-C] bond would shift the active intermediate to 6β-hydroxytestosterone and therefore any 6-delta t formed during the reaction would originate almost solely from molecules of testosterone that lacked $^{3}$H in the 7α position.

Naphthalene metabolism - Buckpitt *et al*. have discovered that naphthalene causes a lesion in pulmonary bronchiolar epithelium of mice but not of rats or hamsters and offered evidence suggesting that the lesion was caused by a

chemically reactive metabolite, presumably a naphthalene-1,2-oxide. However, naphthalene-1,2-oxide may exist in two enantiomers, and thus it seemed possible that the toxicity may depend predominantly on the formation of only one of them. In accord with this view, Buckpitt *et al*. found that in the presence of glutathione and a mixture of glutathione transferases naphthalene was metabolized by liver microsomes from various organs and species to three different glutathione conjugates. They have subsequently identified these conjugates as: 1) 1(S)-hydroxy-2(S)-glutathion-S-yl-1,2-dihydronaphthalene; 2) 1(R)-hydroxy-2R-glutathione-S-yl-1,2-dihydronaphthalene; and 3) 1R-glutathion-S-yl - 2(R)-hydroxy-1, 2-dihydronaphthalene. They also proved that conjugates (1) and (3) were formed solely from naphthalene - 1(S), 2(R)-oxide (designated oxide (1)) whereas conjugate (2) was formed solely from naphthalene - 1(R), 2(S)-oxide (designated oxide (2)).

Last year we reported the isolation from mouse liver of two isozymes of cytochrome P-450, designated as cytochrome $P\text{-}450_N$ and cytochronme $P\text{-}450_S$. During the past year we have found that these isozymes, supplemented with NADPH, GSH and a mixture of glutathione transferases, metabolize naphthalene to markedly different patterns of glutathione conjugates. In view of the findings of Buckpitt *et al*., we conclude that cytochrome $P\text{-}450_N$ forms about 25 times as much oxide (2) as it does oxide (1), whereas cytochrome $P\text{-}450_S$ forms about twice as much oxide (1) as it does oxide (2). Moreover, kinetic studies with microsomes from liver and lung of mice suggest that mouse lung forms about 9 times as much oxide (2) as oxide (1) whereas mouse liver forms about twice as much oxide (2) as it does oxide (1).

## Pharmacokinetics of Short-lived Chenmically Reactive Metabolite *In Vivo*

During the past few years, we have developed rather simple equations that provide estimates of the *in vivo* activities of enzymes in individual organs that catalyze the formation of short-lived chemically reactive metabolite ($t\ 0.5 < 10$ sec.). These equations provide ways of comparing data obtained from *in vivo* and *in vitro* experiments for consistency and permit simulation of predicted values at different doses of precursors of the chemically reactive metabolites. In the past we have focused attention on the relationship between the pharmacokinetics of precursors of chemically reactive metabolites with the kinetics of drug induced depletion and repletion of endogenous substances, such as glutathione, which reacts with the chemically reactive metabolite. These equations require knowledge of the total area under the curve of the precursor in blood and the total area under the curve of the drug-induced loss of the endogenous substances in the organs. During the past year we have developed equations based on the partial areas under the curves (from $t = 0$ to $t = t$) of the precursor in blood and the endogenous substances in organs. The two approaches have been applied to studies of acetaminophen-induced loss of glutathione in hamster liver with excellent results.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00937-05 LCP

PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Mechanisms of mast cell degranulation: PI breakdown and calcium signal

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

Jose Cunha-Melo Vist.Assoc. LCP NHLBI

Other Investigator:
Michael A. Beaven Section Chief LCP NHLBI

COOPERATING UNITS (if any)

N.M. Dean, DCT, NCI; J.L. Barker, LNP, NINCDS, S.V.P. Jones, LNP, NINCDS, J. Moyer, DCT, NCI

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

| 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.)


Antigen-mediated stimulation of hydrolysis of inositol phospholipids in RBL-2H3 cells is dependent on the extent and rate of aggregation of the plasma membrane receptors for IgE. The hydrolysis is associated with an increase in concentration of free Ca2+ [Ca2+]i and histamine secretion. The pattern of phosphoinositide response and dependency on external calcium varies markedly with the type of stimulatory legand used. With the exception of aggregated ovalbumin some stimulation of hydrolysis of inositol phospholipids and small increases in [Ca2+]i were induced by DNP24BSA or higher oligomers of IgE. Whatever receptor aggregation system is employed, the mobilization of intracellular calcium is an insufficient stimulus for secretion. All stimulants at optimal concentrations produced comparable responses in the presence of calcium. The addition of calcium to the medium markedly amplifies the phosphoinositide response and causes the release of histamine. Separation of the inositol phosphate by HPLC has shown that upon stimulation with antigen at least eleven inositol phosphate are produced. The metabolic pathway involved in the production of the various inositol phosphates, as determined by studies with cell extracts, was as follows: Inositol 1,4,5-trisphosphate is converted to inositol 1,4-bisphosphate (subsequently to inositol 1 monophosphate) and to inositol 1,3,4,5-tetrakisphosphate. Inositol 1,3,4,5-tetrakisphosphate was converted back to inositol 1,4,5-trisphospate and to inositol 1,3,4-trisphosphate which, in turn, was degraded to inositol 3,4-bisphosphate and to a lesser extent inositol 1,3-bisphosphate. These were subsequently degraded to inositol 1- or 4-monophosphate. In studies with patch clamped single cells, both inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate when injected into cells activated the same kind of ion-conducting channels as those observed with antigen stimulation.




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

Project Description:

Objectives: To establish the metabolic pathway of generation and degradation of water soluble inositol phosphates in antigen stimulated RBL 2H3 cells and to determine the physiological activities of the inositol phosphates in single RBL-2H3 cells

Methods Employed: Cell culture procedures and techniques for measurement of $[Ca_i^{2+}]$ PI breakdown and histamine release have been described previously (J.Biol. Chem. 259:7129 and 7137, 1984). The cellular pools of phosphoinositides were labeled by overnight incubation of cells with $^3$H-labeled inositol. The parent membrane phospholipids; phosphotidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP) and phosphatidylinositol 4,5-bisphosphate ($PIP_2$) were separated from their water soluble cleavage products, inositol 1-monophosphate (IP), inositol 1,4-bisphosphate ($IP_2$) and inositol 1,4,5-trisphosphate ($IP_3$) by solvent extraction. The individual phosphoinositides and inositol phosphates were separated by chromatography on thin layer plates and by high pressure liquid chromatography (HPLC) (Biochem. J. 242:36,1987). Cultures were stimulated (after priming with the appropriate antigen-specific IgE) with aggregated ovalbumin and dinitrophenol conjugated with bovine serum albumin ($DNP_{24}BSA$) or, in unprimed cultures, with covalently cross-linked oligomers of IgE as described in previous reports.

Variously labeled inositol phosphates were prepared from commercially available labeled inositol phosphates ($^3$H and $^{32}$P) by selective incorporation of [$^{32}$P] phosphate into the 3 position by inubation with a rat brain inositol 1,4,5-trisphosphate 3'kinase; and by enzymatic degradation of [$^3$H]inositol 1,3,4,5-tetrakisphosphate followed by purification of the individual metabolic products on HPLC.

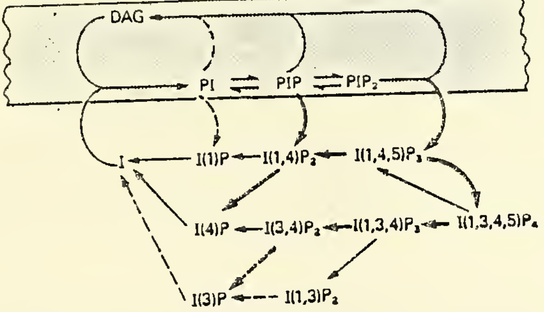
Major Findings: Intact cells: We have re-examined by HPLC the hydrolysis of $^3$H-labeled inositol phospholipids in rat basophilic leukemia (RBL-2H3) cells. Previous studies showed no clear responses between the release of any particular inositol metabolite and the calcium signal in these cells. Paradoxically no cerborises were observed when the cells were stimulated with the antigen aggregated ovalbumin, in the absence of釋 externol calcium (JBC 259:7129,1984 and 261:2583,1986). However, aggregation of the IgE receptors by agents other than aggregated ovalbumin caused the release of small amounts of [$^3$HO]inositol phosphates and a small increase in levels of cytosol $Ca^{2+}$($\sim$25 nM). The response varied with the type of stimulant used. Within seconds after addition of the antigen $DNP_{24}BSA$ there was a small burst in release of [$^3$H]inositol 1,4,5-trisphosphate, [$^3$H]inositol 1,3,4,5-tetrakisphosphate and [$^3$H]inositol 1,3,4-trisphosphate which was followed by a gradual rise in inositol 1,3,4-trisphosphate, inositol trisphosphate and inositol monophosphate. Eventually all inositol phosphates reached different steady-state levels which were maintained for at least 40 min. In contrast, the initial response to oligomeric IgE, which aggregates receptors at a relatively slow rate, was muted although the subsequent development of the response was the same. The levels of inositol pentakisphosphate and hexokisphosphate remained unchanged.

The finding of additional isomers of inositol phosphates by HPLC techniques. At least 3 isomers of the inositol monophosphate and inositol bisphosphate were apparent in extracts of RBL-2H3 cells stimulated by antigen. Although, in the presence of Li+, the rate of accumulation of all inositol phosphate, except $IP_5$ and $IP_6$, were correlated with the calcium signal there was no exact correlation between the appearance or disappearance of any one metabolite and the calcium signal. The possibility existed that the calcium signal may be generated by more than one mechanism but may be initally dependent on mobilization of intracellular calcium.

Determination of metabolic pathways in studies with cell extracts. The studies with cell extracts and variously labeled inositol phosphates demonstrated that 2H3 cells contain the enzymes that catalyze all of the metabolic transformations observed when the intact cells were stimulated with antigen. These included the rapid conversion of inositol 1,4,5-trisphosphate to inositol 1,3,4,5-tetrakisphosphate and subsequent formation of inositol 1,3,4-trisphosphate, as well as the dephosphorylation of inositol 1,4,5 and 1,3,4-trisphosphate to yield the inositol 1,4-trisphosphate, 1,3-bisphosphate and 3,4-trisphosphate. Subsequently the bisphosphates were degraded to inositol 1,3 and 4 monophosphate and thence inositol.

The dephosphorylation reactions were catalyzed by both soluble and membrane associated phosphatases, the most predominant membrane bound phosphatase being the 5-phosphomonoesterase. An unexpected finding was that [$^3$H]inositol 1,3,4,5-tetrakisphosphate when incubated with extracts of RBL-2H3 cells was converted to two isomers of [$^3$H] inositol trisphosphate, identified as I(1,4,5) trisphosphate and [$^3$H]inositol 1,3,4-trisphosphate. The degradation of these two metabolites yielded the expected 1,3-bisphosphate, 1,4-bisphosphate and 3,4-bisphosphate of [$^3$H]inositol.

The exact identity of these metabolites was confirmed by 1) co-elution with previously characterized standards, 2) loss of specific [$^{32}$P] phosphate group (i.e. 3,4 or 5 phosphate) and by the pattern of metabolism obtained by incubation with phosphatase enzyme activities. The pathway of metabolism was found to be as follows:




Studes by patch clamp techniques. Membrane conductance were investigated using the whole cell patch-clamp technique with pipettes containing (in mM) 150 KCl, 0.1 CaCl, 1.1 EGTA, 2 MgCl and 5 HEPES (free calcium concentration 10 nM). The cells were primed with IgE (5 µg/ml) and voltage-clamped at - 60 mV. Prior to antigen stimulation the only demonstrable conductance was a cesium-sensitive inward rectifier, which was activated at potentials more hyperpolarized than - 60 mV. However, on stimulation with antigen (DNP/BSA 10 ng/ml) a low amplitude inward current developed, which peaked at 10 min and then declined to pre-stimulation

levels 30 min after application of antigen. The time course of this inward current was paralleled by that of a voltage-independent outward current. Both of these currents could be activated in the absence of antigen stimulation when inositol 1,4,5-trisphosphate ($IP_3$) or inositol 1,3,4,5-tetrakisphosphate ($IP_4$) (10 μM) was included in the patch pipette solution. Thus, intracellular levels of $IP_3$ and $IP_4$ can mimic antigenically stimulated changes in membrane excitability. From these results we conclude that the antigen-antibody interaction increases intracellular $IP_3$ which liberates calcium from intracellular stores, and this in turn may activate calcium-dependent currents.

Significance of Biomedical Research and to the Program of the Institute. The studies delineate all of the pathways for metabolism of inositol phosphates that are released into the cytosol of antigen-stimulated RBL-2H3 cells. This has led to the discovery of novel pathways of phosphoinositide metabolism. The studies with patched clamped cells provide enticing evidence that inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate act as second messengers, possibly in concert with changes in $[Ca^{2+}]_i$; to activate the opening of ion channels in the plasma-membrane of RBL-2H3 cells. Other studies (Project Report No. Z01 HL 00990-01 LCP) indicate that enough inositol 1,4,5-trisphosphate is produced in antigen-stimulated RBL-2H3 cells to activate $Ca^{2+}$-release mechanisms within the cell. The projects collectively have now established the credentials of inositol 1,4,5-trisphosphate (and possibly inositol 1,3,4,5-tetrakisphosphate) as a second messenger in IgE-receptor mediated secretion. They indicate, in addition, that degradation of the messenger(s) is rapid and proceeds through multiple pathways.

Proposed Course of Project: As the major goals of the project have been achieved, future studies will focus on events at the membrane-level in studies with permeabilized and patch-clamped cells. The project is terminated.

Publications:

Beaven, M.A.: Beyond the receptor. How stimulatory signals are transmitted in the cells; in Development of Drugs and Modern Medicine (Gorrod, J., Gibson, G.G. and Mitchard, M. (Eds.).), Ellis Horwood Publishers, Chichester, pp. 107-116, 1986.

WoldeMussie, E., Maeyama, K. and Beaven, M.A.: Loss of secretory response of rat basophilic leukeia (2H3) cells at 40° is due to reversible suppression of intracellular signals as indicated by inositol phospholipid breakdown and increases in cytosol $Ca^{2+}$. J. Immunol., 137: 1674-1680, 1986.

Cunha-Melo, J.R., Dean, N.M., Moyer, J.D., and Beaven, M.A.: Kinetics of phosphoinositide hydrolysis in rat basophilic leukemia (RBL-2H3) cells varies with the type of IgE - receptor cross agent used. J. Biol. Chem., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00962-05 LCP

PERIOD COVERED

October 1, 1986 to September 30, 1987

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. J. Gerald Kenna | Guest Researcher | LCP | NHLBI |
| Other Investigators: | | | |
| Lance R. Pohl | Section Chief | LCP | NHLBI |
| Hiroko Satoh | Vist. Assoc. | LCP | NHLBI |
| David D. Christ | Guest Researcher | LCP | NHLBI |

COOPERATING UNITS (if any)

William Kammerer, Anesthesiology Section, Clinical Center, NIH

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Pharmacological Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892


| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.25 | 1.25 | |

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.)


My previous work, performed at the Liver Unit, King's College Hospital, (KCH), London U.K., has demonstrated that sera from patients with halothane-hepatitis react with several halothane induced liver microsomal polypeptide antigens not expressed in normal liver. Antibodies to these antigens are specific to such patients and are not produced simply as a consequence of halothane exposure and/or liver damage, indicating that they may play a role in development of halothane hepatitis in vivo. We have now demonstrated that the antigenic sites recognized by the patients antibodies consist of a specific halothane metabolite hapten, the trifluoroacetyl group (TFA-), bound to endogenous liver proteins. These results constitute the first demonstration of an immune response in patients with drug toxicity directed against novel antigens produced by a defined covalently bound metabolite. Purification of the protein antigens is currently in progress, using affinity chromatography methods developed previously in this Laboratory for the purification of trifluoroacetylated proteins. The purified proteins will be used in animal model studies, and also for their detailed biochemical characterization.




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

Project No. Z01 HL 00962-05 LCP

Project Description:

Objectives: Determination of the molecular events underlying the severe hepatic necrosis caused by the anesthetic agent halothane (halothane hepatitis).

Methods Employed: Four to 12 hr following ip administration of halothane or deuterated halothane to uninduced or phenobarbital (PB) induced rats, liver microsomal fractions were prepared and tested by immunoblotting for the presence of halothane induced polypeptide antigens reacting with antibodies in sera from patients with halothane hepatitis, and with an antibody to the halothane metabolite hapten N-epsilon-TFA-L-lysine. Liver microsomal fractions from $CCl_4$ pretreated rats were also tested by immunoblotting for reaction with the antibodies. Halothane was incubated with control rat liver microsomes *in vitro*, and the effects of varying $O_2$ tensions and additions of NADH and NADPH upon generation of the antigens were determined. The ability of TFA-lysine as compared with acetyllysine to inhibit competitively antibody binding to the antigens, and also the effect of chemical cleavage of TFA- groups from the microsomal proteins using 1M piperidine, was investigated.

Major Findings: Administration of halothane to rats was found to result in expression of halothane induced polypeptide antigens, recognized by antibodies in sera from patients with halothane hepatitis, of identical apparent molecular mass to those identified previously in rabbits and humans exposed to the drug. Oxidative metabolism by cytochrome P-450, which produces the reactive species $CF_3COX$ which in turn binds covalently to proteins, was required for antigen expression. Using a specific anti-TFA- antibody, the polypeptides recognized by the patients' antibodies (100 kDa, 76 dKa, 58 kDa, 57 kDa, 54 kDa) were found to contain covalently bound TFA- groups. Binding of the patient's antibodies was inefficiently blocked by addition of the hapten inhibitor TFA-lysine, but was efficiently abolished by chemical removal of TFA- groups with 1 M piperidine. Thus the patient's antibodies recognized novel antigenic sites consisting of TFA- bound to endogenous liver proteins, and apparently react with a combination of TFA- plus peptide backbone, and not simply TFA- alone.

Significance to Biomedical Research and Program of the Institute: Although many examples of idiosyncratic drug toxicity are thought to have an immune basis, very little mechanistic information is currently available. Our focus of interest is halothane hepatitis. We anticipate that the molecular events involved, and the methodologies developed in this study, should be of general applicability to other forms of immune mediated drug toxicities. My previous research, performed in the Liver Unit, KCH, London, UK, has demonstrated antibodies to several novel halothane induced liver polypeptide antigens in sera from patients with halothane hepatitis. We have now demonstrated that these antibodies recognize a specific halothane metabolite, produced by cytochrome P-450 mediated metabolism of the drug, bound covalently to liver proteins. It has been suggested previously that immune sensitization to drug metabolites bound to tissues would be a possible way for a chemically inert compound such as halothane to provoke tissue specific immunotoxicity. Our results constitute the first demonstration of an immune response in patients with drug toxicity to novel antigens so generated.

We have demonstrated that the novel protein antigens recognized by the patients' antibodies contain covalently bound TFA-. Methods have been developed in this Laboratory for purification of such proteins using an anti-(TFA-) antibody affinity column, therefore purification of the antigens should now be possible. Purified proteins can be used to immunize animals, followed by challenge with halothane, in an attempt to develop an immune mediated animal model of the toxicity, and prove that immune reactions to the antigens are involved pathogenically in development of halothane hepatitis. Antigen purification should help us also to biochemically characterize the proteins, and in particular to determine their function in the hepatocyte, and whether they correspond to known liver proteins.

Also, the fact that the protein antigens have become labeled by TFA-, a very reactive species produced by cytochromes P-450, suggests that the proteins may be either associated with, or situated very close to, cytochromes P-450 in the microsomal membrane. Further studies of the mechanism of TFA-labeling of the proteins should thus provide valuable information concerning interactions between the proteins of the microsomal membrane.

Proposed Course of Project: We intend to: 1) Purify the protein antigens using anti-TFA-affinity chromatography, and produce monospecific antibodies to them. 2) Biochemically characterize the structural features of the protein antigens, determine their tissue distributions and subcellular locations, and investigate possible identities with known liver proteins. 3) Develop an animal model by active immunization of animals with the purified antigens, or passive immunization with specific antibodies raised using purified antibodies, then challenge with halothane. 4) Determine whether the protein antigens are associated with cytochrome P-450 in the microsomal membrane by cross-linking experiments and studies of the time course and temperature dependence of their labeling by TFA- *in vitro*.

Publications:

Satoh, H., Gillette, J.R., Takemura, T., Ferrans, V.J., Jelenich, S.E., Kenna, J.G., Neuberger, J. and Pohl, L.R.: Investigation of the immunological basis of halothane-induced hepatotoxicity in Biological Reactive Intermediates III (Snyder, Jollow, Kocsis and Nelson, eds.) Plenum Press, 1986, 657-673.

Satoh, H., Davies, H.W., Takemura, T., Media, K., Gillette, J.R. and Pohl, L.R.: An immunochemical approach to investigating the mechanism of halothane-induced hepatotoxicity, in Progress in Drug Metabolism, Vol. 10, J.W. Bridges and L.F. Chasseaud (eds.) pp. 187-206, Taylor and Francis Ltd. (1987).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00967-05 LCP

PERIOD COVERED

October 1, 1986 through September 30, 1987

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*)

| | Name | Title | Lab | Institute |
|---|---|---|---|---|
| P.I. | Helen W. Davies | Staff Fellow | LCP | NHLBI |
| Others: | Lance R. Pohl | Section Chief | LCP | NHLBI |
| | John W. George | Chemist | LCP | NHLBI |

COOPERATING UNITS (*if any*)

Dr. Pedi Neta, National Bureau of Standards

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Pharmacological Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.75 | 1.25 | 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.*)

We have previously reported that the suicide inactivation of cytochrome P-450 by many structurally diverse drugs and environmental chemicals is caused by a novel pathway which involves the irreversible binding of products derived from the heme prosthetic group to the protein moiety of the enzyme. The pathway for the destruction appears to be a general one involving the initial metabolism of the chemical or drug by cytochrome P-450 into a radical intermediate, which reacts with the heme prosthetic group, leading to its chemical activation. Most likely the reactive heme intermediate contains a radical or cationic center, which can then be attacked by an amino residue(s) of the protein moiety. If the protein is not catalytically inactivated by this first covalent event, the process can be repeated until the catalytic activity of the enzyme is lost. This process may explain how other heme enzymes such as prostaglandin H synthase, prostacylin synthase, and thromboxane synthase are inactivated during their normal catalytic turnovers or by the hydroperoxides in general.



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

Project Description:

Objectives: To determine the mechanisms of drug-induced inactivation and heme and apoprotein turnover of cytochrome P-450.

Methods Employed: Liver microsomes were prepared from phenobarbital-induced male Sprague-Dawley rats which had been treated with [3,5-$^3$H] delta aminolevulinic acid to radiolabel microsomal heme. Radiolabeled ($^3$H-heme moiety) cytochrome P-450 (54 kD) and cytochrome P-450 reductase were purified by published procedures. The inactivation of [$^3$H]-labeled cytochrome P-450 by [$^{14}$C]-$CCl_4$ was studied in reconstituted enzyme mixtures containing purified cytochrome P-450, cytochrome P-450 reductase, dilauroylphosphatidylcholine and an NADPH generating system. Model heme degradation studies were performed by irradiating a mixture of myoglobin, phosphate buffer (pH 7.4), isopropranol, and $CCl_4$ in a cobalt 59 source (gamma radiolysis) at the National Bureau of Standards. Following the incubations, reaction mixtures were hydrolyzed enzymatically with proteinase K and the products were analyzed by HPLC.

Major Findings: When cytochrme P-450 was incubated in a reconstituted enzyme system with $CCl_4$ up to 96% of the cytochrome P-450 heme was destroyed. As much as 49% of the destroyed heme could be accounted for as heme-derived products irreversibly bound to protein. By separation of the reaction mixtures by SDS/PAGE, it was demonstrated that the heme-derived products were bound exclusively to cytochrome P-450 protein, and not to cytochrome P-450 reductase. The visible absorption spectrum of the reaction mixture showed nearly the complete loss of the Soret absorption band at approximately 400 nm, suggesting that the conjugated heme ring system was completely disrupted. Products from the enzyme hydrolysis of the raction mixture were separated by reverse phase HPLC. Further analysis and purification of these fractions by a variety of HPLC procedures revealed that the products containing heme-derived [$^3$H] radiolabeled always contained the [$^{14}$C] radiolabeled derived from $CCl_4$. Two hydrolysis products were eventually purified. Both were shown by sizing column analysis to have molecular weights of less than 2000 therefore are likely to be derivatives of single amino acids or of small peptides. Based on the specific activities of the $CCl_4$ and heme initially present in the reconstitution mixture, the molar ratios of $CCl_4$ to heme in the two purified products were 4-5:1 and 9-10:1. In contrast, the model reaction mixtures, in which myoglobin was reacted with a $CCl_3$ radical generated by gamma radiolysis, showed virtually no loss of the intensity of the Soret absorption, even though the heme moiety was found to be attached covalently to the protein by precipitation of the reaction mixtures with acetone-HCl, conditions which release the heme group from myoglobin. Hydrolysis of the reaction mixtures with Proteinase K followed by HPLC analyses revealed the presence of at least two amino acid residues containing the covalently bound heme-derived moiety or moieties.

Significance to Biomedical Research and Program of the Institute: The results during this year have clearly shown that the inactivation of cytochrome P-450 by many drugs and various environmental chemicals is due to the covalent binding of products of the heme prosthetic group to the protein moiety of the enzyme. The above labeling studies with $CCl_4$ suggest that destruction process is initiated by a radical metabolite produced at the active site, which reacts with the heme prosthetic group, leading to

its chemical activation. Most likely the reactive heme intermediate contains a radical or cationic center, which can then be attacked by an amino residue(s) of the protein moiety. If the protein is not catalytically inactivated by this first covalent event, the process can be repeated until the catalytic activity of the enzyme is lost. This appears to be the case for the destruction by $CCl_4$ inasmuch as several carbon atoms from $CCl_4$ appear to bound to each mole of covalently bound heme-derived product. The model studies with myoglobin are important for at least two reasons. First, they clearly indicate that radical products near the heme prosthetic group of any heme containing enzyme may lead to the covalent binding of the heme prosthetic group to the protein moiety. This process may explain how other heme enzymes such as prostagalndin H synthase, prostacylin synthase, and thromboxane synthase are inactivated during their normal catalytic turnovers or by hydroperoxides in general. Secondly, because the covalently bound heme-derived moiety of myoglobin appears to contain an intact tetrapyrrole structure, the process leading to its formation may model the initial covalent event that occurs during the inactivation of other heme protein.

Proposed Course of Project: In order to better understand the mechanism of the inactivation of cytochrome P-450 by the xenbiotics studies to date and to aid in final characterization of the structures of the P-450 heme adducts, studies will be continued using myoglobin as a model heme protein. Myoglobin has the advantages of having a known structure, being readily available in large quantities, and existing as several well defined structural variants. Identification of the structure of the heme derived amino acid adducts from the myoglobin reactions should provide both a model for and standards for completion of the cytochrome P-450 studies as well as studies of the mechanism of inactivation of other heme proteins.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00973-03 LCP

PERIOD COVERED

October 1, 1986 to September 30, 1987

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)


Jose Cunha-Melo | Visiting Assoc. | LCP | NHLBI

Other Investigators:

Michael A. Beaven | Section Chief | LCP | NHLBI


COOPERATING UNITS (if any)

Drs. K.P. Huang and F. Huang, ERRB, NCHHD

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.4 | 0.4 | |

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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.)


Aggregation of IgE receptors on RBL-2H3 cells with oligomers of IgE and activation of regulatory GTP binding proteins by sodium fluoride induced substantial release of inositol phosphates whereas the ionophores A23187 and the Na+ ionophore, monensin, induced only limited release of the sugar phosphates. All of the above reagents stimulated histamine release to a variable extent with monensin inducing the least release. Oligomers of IgE and A23187 caused large increases in concentration of cytosol Ca2+ (Ca2+)i, sodium fluoride produced a slow but progressive increase in [Ca2+]i over the course of 30 min. Monensin produced no perturbation in the levels of [Ca2+]i. In contrast to the above agents the phorbol ester, PMA, elicited neither stimulatory nor secretory responses in 2H3 cells. Of the various combinations of drugs tested the most marked effect was the enhancement of breakdown of phospholipids and calcium signal when sodium fluoride and monensin were tested in combination and enhancement of histamine release when cells were exposed to combination of PMA and A23187. Also stimulation of breakdown of phospholipids by both oligomers and sodium fluoride was markedly reduced in cells exposed to short-term exposure to PMA. The secretory response to sodium fluoride and A23187 but not to higher oligomer was enhanced markedly by short-term response to PMA. In contrast to short-term exposure to PMA, after prolonged exposure (20 hrs) to PMA both the inhibitory effects on release of inositol phosphates and synergistic effects on secretion of histamine were lost. These data suggested that protein kinase C exerted both stimulatory and inhibitory effects on signal transduction and that its modulatory actions were lost with long-term exposure to PMA. Tests with polyclonal antibodies against protein kinase C indicated that long term treatment of cells with PMA resulted in disappearance of protein kinase C from both cytosol and membrane.




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

Project Description:

Objectives: Antigen-induced secretion in the rat basophilic (RBL-2H3) cell line is mediated by hydrolysis of membrane inositol phospholipids (see Project Report No. Z01 HL 00937-04 LCP). This hydrolysis results in the generation of two messengers, inositol 1,4,5-trisphosphate and diacylglycerol which induce, respectively, the mobilization of $Ca^{2+}$ ions and the activation of protein kinase C. The mobilization of $Ca^{2+}$ ions occurs largely through influx of $Ca^{2+}$ ions across the plasma membrane (J. Biol. Chem. 259: 7129, 1984) possibly through the opening of ion channels (see Project Report No. Z01 HL 00937-04 LCP). The evidence for the activation of protein kinase C in RBL-2H3 cells is the phosphorylation of protein kinase C - target sites on the light chain of myosin (see Project Report No. Z01 HL 0099993-01 LCP). The time-course of phosphorylation and increase in levels of cytosol $Ca^{2+}$ ($[Ca^{2+}]_i$ correlate with that of histamine secretion.

As reported in the previous Project Reports, studies with the phorbol ester, phorbol myristic acid (PMA), indicate that activation of protein kinase C (wtih PMA) promote synergistic signals for secretion and, at the same time, down-regulates stimulatory events (i.e. hydrolysis of inositol phospholipids) triggered by receptor-aggregation.

Other studies, that are described in accompanying projects, present evidence that a GTP-binding protein (G-protein) facilitates coupling of the receptor to the catalytic unit, phospholipase C. The evidence is based on the stimulatory effects of activators of G-protein (i.e. GTP S in permeabilized cells and sodium fluoride in intact cells) on the parental RBL-2H3 cell.

In the present project the idea that protein kinase C has both a stimulatory and regulatory role in signal transduction was subjected to critical examination. This was made possible by our finding that prolonged treatment of RBL-2H3 cells results in the disappearance of protein kinase C. To determine whether or not "down-regulation" is exerted through the G-protein three levels of stimulation were employed, 1) aggregation of IgE receptors with oligomers of IgE; 2) use of stimulants of G-proteins and 3) the $Ca^{2+}$ ionophore A 23187 which, by inducing large increases in $[Ca^{2+}]_i$, stimulates phospholipase C directly (Lo et al., J. Biol. Chem. 262:4141,1987).

Methods Employed: All procedures were performed as described in previous publications (J. Biol.Chem. 259:7129 and 7137,1984). The demonstration of protein kinase C in cells was done as described by Huang (J. Biol. Chem. 261: 14781,1987. Cells were permeabilized by streptolysin O as described in Project Report No. Z01 HL 00990-01 LCP).

Major Findings: Substantial hydrolysis of inositol phospholipids and secretion of histamine was induced by treatment with oligomers and NaF. The stimulatory effect of the latter compound was enhanced by the $Na^+$-ionophore, monensin, presumably by facilitating the uptake of NaF. As described in other reports, A23187, at high concentrations (200-1,000 nM) induced a $Ca^{2+}$-dependent breakdown of inositol phospholipids. Stimulation with oligomers and A23187 resulted in prompt and large increases in $[Ca^{2+}]_i$ whereas NaF elicited a slow increase in $[Ca^{2+}]_i$ over the course of 30 min. Low concentrations of A23187 ($< 100$ nM) did not induce hydrolysis of the phospholipids or secretion of histamine but still

caused increases in $[Ca^{2+}]_i$ in concentrations down to 10 nM. PMA by itself induced neither stimulatory or secretory responses.

Effect of short-term treatment with PMA (50 nM for 10 min). Such treatment resulted in marked potentiation of secretion induced by A23187 and to a lesser extent by NaF. Indeed substantial secretion was observed with combinations of A23187 (25 to 100 nM) and PMA (10 to 50 nM) which by themselves elicited no no response. Secretion caused by the oligomers was not markedly affected in cells treated with PMA.

In contrast to the secretory response, hydrolysis of inositol phospholipids was depressed in a concentration-dependent fashion by PMA when cells were stimulated with oligomers or NaF but not with A23187. The increase in $[Ca^{2+}]_i$ in response to oligomers and NaF (but not A23187) was also suppressed by treatment with PMA.

Effect of long-term treatment with PMA (50 nM for 20 hr). After prolonged treatment with PMA, the stimulatory response (i.e. hydrolysis of inositol phospholipids) to all stimulants was restored (i.e. the same as untreated cells) but the secretory response was markedly impaired (e.g. by 77% for the oligomers) and the potentiating (and synergistic) interactions between PMA and A23187 (and NaF) were no longer observed.

Examination of Western immunoblots (with polyclonal antibody) showed that upon exposure of RBL-2H3 cells to PMA, the protein kinase C that was initially bound to the plasma membrane disappeared over the course of several hours. By 6 hr almost all of the intact (Mol.Wt. 82 kDa) protein kinase C had been cleaved initially into two fragments one of which was identified as the PMA-binding fragment (the regulatory subunit) and the other as the kinase fragment. By 24 hr almost all of the immunologically recognizable fragments had disappeared from the cytosol. The loss of protein kinase C was consistent with the loss of synergistic and inhibitory actions of PMA noted above.

Significance to Biomedical Research and the Program of the Institute: The studies provide strong evidence that protein kinase C exerts both stimulatory and inhibitory actions following receptor aggregation in RBL-2H3 cells. As the inhibitory actions are expressed when cells are stimulated with oligomers and NaF (but not with A23187) the inhibitory actions of PMA are probably exerted through the phosphorylation of a G-protein.

Proposed Course of Project: As preliminary studies indicate that the inhibitory actions of PMA are lost once RBL-2H3 cells are permeabilized, experiments will be undertaken to determine the residual levels of protein kinase C in the permeabilized cells. If the enzyme is lost, the effect of adding back purified protein kinase C will tested. Once the G-protein that couples to phospholipase C has been identified (see Project Report No. Z01 HL 00975-03 LCP), the possibility that this G-protein is phosphorylated in response to PMA treatment will be examined.

Publications:

Mizuguchi, J., Beaven, M.A., Hu-Li, J., and Paul, W.E.: Phorbol myristate acetate inhibits anti-IgG mediated signalling in resting B. cells. Proc. Natl. Acad. Sci. (NY), 83: 4474-4478, 1986.

Mizuguchi, J., Tsang, W., Morrison, S.L., Beaven, M.A. and Paul, W.E.: Membrane IgM, IgD and IgG act as signal transmission molecules in a series of B. lymphomas. J. Immunol. 137: 2162-2167, 1986.

Mizuguchi, J., Beaven, M.A., Ohara, J. and Paul, W.E.: BSF-1 action on resting B. cells does not require elevation of inositol phospholipid metabolism or increased $[Ca^{2+}]_i$. J. Immunol. 137: 2215-2219, 1986.

Paul, W.D., Mizuguchi, J., Beaven, M.A., Hornbeck, P., Tsang, W. and Ohara, J.: B. lymphocyte activation. The roles of receptor cross-linkage and BSF-1. Proceedings of the lymphocyte activation and immune regulation meeting, Plenum Press, NY, in press.

Mizuguchi, J., Beaven, M.A., Hornbeck, P., Tsang, W. and Paul, W.D.: Receptor cross-linkage stimulates B cell activation in Antibodies - structure, synthesis, function and immunologic intervention in disease, Eds. Szentwanyi, A., Mauer, P. and Janicki, B.W. Plenum Press, NY, in press.

Beaven, M.A., Guthrie, D.F., Moore, J.P., Smith, G.A., Hesketh, T.R., Metcalfe, J.C.: Synergistic signals in the mechanism of antigen-induced exocytosis in 2H3 cells. Evidence for an unidentified signal required for histamine release. J. Cell Biol., in press.

Mizuguchi, J., Yong-Yong, J., Nakabayaschi, H., Huang, K.P., Beaven, M.A., Chused, T. and Paul, W.E.: Protein kinase C activation blocks anti-IgM-mediated signalling in BAL 17 lymphoma cells. J. Immunol., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00975-03 LCP

PERIOD COVERED

October 1, 1986 through September 30, 1987

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. Hydar Ali — Vist. Fellow — LCP — NHLBI

Other Investigator:
Michael A. Beaven — Deputy Chief — LCP — NHLBI


COOPERATING UNITS (if any)

Dr. Joel Moss, LCM, NHLBI and Dr. Reuben Siraganian, National Institute of Dental Research

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

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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.)


Antigen mediated histamine release from RBL-2H3 cells is associated with substantial hydrolysis of membrane inositiol phospholipids and an increase in cytosol $Ca^{2+}$ (Ca signal). As reported last year, studies with most variants of the RBL-2H3 cells revealed a correlation between hydrolysis of inositol phospholipids, Ca signal and histamine secretion in response to antigen stimulation. One variant, TG-1B3, however, showed no Ca signal as measured by quin-2, fura-2 and $^{45}Ca^{2+}$-uptake but still showed modest hydrolysis of inositol phospholipid and histamine release. Several variants were completely unresponsive to antigen but did release histamine when challenged with a combination of ionophore and phorbol ester. Since these variants possessed normal numbers of receptors for IgE, and had abundant phospholipase C activity, the possibility of defective coupling between receptor aggregation and activation of phospholipase C was investigated. One variant BUDR-2B1, showed no or very little phosphoinositide hydrolysis in response to stimulants of GTP-regulatory proteins such as sodium fluoride in intact cells and GTP S in permeabilized cells. All other unresponsive variants did respond to these stimulants. In preliminary studies, the isolation, purification and hybridization of mRNA with specific radiolabeled probes for two GTP-regulatory proteins, Gi and Go, revealed that both the variants and the parental 2H3-cells transcribed message for Go but not for Gi.

Project Description:

Objectives: Stimulation of RBL-2H3 cells by aggregation of receptors for IgE with antigen or with covalently cross-linked oligomers of IgE leads to production of inositol phosphates and diacylglycerol. At least two stimulatory signals are thought to be generated by this reaction. A $Ca^{2+}$ signal, which is generated primarily through translocation of $Ca^{2+}$ ions across the plasma membrane due possibly to the production of inositol phosphates. The second signal is the activation of protein kinase C by diacylglycerol. As indicated by studies with $Ca^{2+}$ specific ionophores and activators of protein kinase C both signals are obligatory for secretion in 2H3 cells. Variants of 2H3 cells showed interesting properties (see last year's report). One TG-1B3, can be stimulated to secrete histamine with antigen or a combination of ionophore and phorbol ester. Although secretion is dependent on external $Ca^{2+}$, antigen-induced secretion occurs without a detectable increase in concentration of cytosol $Ca^{2+}$. Three other variants, contain IgE receptors and, as far as we could determine, all components of the phosphoinositide cycle. They are however, refractory to antigen stimulation.

There is indirect evidence from studies with nonhydrolysable analogues of the guanine nucleotide, GTP, that a GTP-binding protein (G-protein) may allow coupling of receptors to the catalytic unit, phospholipase C. The particular species of G-protein involved has not been identified. Because of the likely possibility that some of the unresponsive RBL-2H3 variants may lack such a protein, we report here studies with stimulants of G-protein in intact and permeabilized cells. In parallel with this approach, the variants were screened for the absence (or presence) of mRNA for specific G-proteins.

Methods Employed: The maintenance of cultures, sensitization of cells with IgE, release experiments with antigen and ionophore/phorbol ester were as described in the previous report. Phospholipase C activities were determined by methods described by Siess and Lapentina (BBA 752:329,1983). Permeabilization of cells by high voltage electric discharge and by streptolysin O were performed as described by Knight and Baker (J.Membrane Biol. 65:107,1982) and Howell and Gomperts (BBA 927:177,1987). Total RNA was prepared by lysis of cells in guanidine isothiocyanate and by ultracentrifugation over a gradient of cesium chloride. The mRNA was purified by a chromatographic technique using an oligo-dt-cellulose column (Methods in Molecular Biology 139:142,1986) and screened for the presence of message for two G-proteins, Gi and Go by hybridization with specific radiolabeled probes.

Major Findings: Evidence for the participation of a G-protein in phosphoinositide response in 2H3 cells and its variants. The presence of IgE receptors, and the ability of ionophore in combination with phorbol ester to stimulate secretion in the antigen unresponsive variants was confirmed in the present study. Sodium fluoride and GTP $\gamma$S were used to test the possibility that GTP-binding protein(s) might be absent or defective in these clones. These two compounds stimulate the hydrolysis of inositol phospholipids in other types of cells, presumably through activation of GTP-regulatory proteins. The response to NaF (20 mM with intact cells) and GTP $\gamma$S (permeabilized electrically, or with streptolysin O was variable.

One defective cell line (BUDR-2B1) failed to respond to either of the two stimulants whereas hydrolysis of the inositol phospholipids was stimulated by these compounds in other defective variants and the parental RBL-2H3 cell line.

In an attempt to identify the particular G-protein(s) that might be absent in the BUDR-2B1 variant and, by implication, the G-protein(s) that is specifically responsible for activation of phospholipase C we isolated and purified mRNA. Hybridization of isolated mRNA from the parental 2H3 line and the variant line (BUDR-2B1) with specific radiolabeled probes for Gi and Go suggested that both the variant and parent line possesses the message for Go but not that for Gi.

Significance to Biomedical Research and to the Program of the Institute: By pharmacological criteria one variant lacks or has a defective coupling protein that links receptor aggregation and activation of phospholipase C. Although several GTP-regulatory proteins have been identified, for which specific functions have not been assigned, the particular G-protein that mediates inositol phospholipid hydrolysis remains unidentified: It has been designated as Np or Gp because its presumed ability to activate phospholipase C. From studies with pertussis toxin in mast cells, a soluble alpha subunit of Gi (the G-protein that inhibits adenylate cyclase) has been implicated (J. Biol. Chem. 260:3584,1985) but our preliminary experiments with mRNA suggests that Gi is absent in RBL cells. The presence of message for Go, on the other hand in both the nonresponsive (BUDR-2B1) variant and the parent 2H3 cells suggest that this G-protein can also be excluded from consideration.

The BUDR-2B1 variant provides good prospects of positive identification of Gp in the near future. If this can be accomplished the variant would be a valuable tool for investigating the mechanism of phospholipase C activation at the molecular level.

Proposed Course of Project: The absence of a phospholipase C - coupling protein(s) in the BUBR-2B1 variant will be explored further by hybridization of purified mRNA with probes for other G-proteins. Antibodies to known forms of coupling proteins will also be used.

The TG-1B3 variant will be critically evaluated from several points of view. The properties of this variant may point to undiscovered aspects of the phosphoinositide signal cascade mechanism. Theoretically this variant should not secrete histamine because of the absence of a detectable $Ca^{2+}$-signal and our inability to detect protein kinase C by polyclonal antibodies (preliminary data).

The studies with this variant will test whether or not; 1) sufficient amounts of inositol 1,4,5-trisphosphate are generated to release intracellular calcium (mass measurement, see Project Report No. Z01 HL 00990-01 LCP); 2) inositol 1,4,5-trisphosphate releases $Ca^{2+}$ from intracellular stores; 3) protein kinase C is absent by measurement of (in permeabilized cells) enzyme activity; and 4) other protein kinase activities (A and G) are activated during antigen stimulation. Options for further studies include the presence of an, as yet, undiscovered family of protein kinase C (3 genetically different families have

been described to date) and the $Ca^{2+}$-dependent processes (e.g. myosin phosphorylation) in this variant operate at or near basal $Ca^{2+}$ levels i.e. 100 nM. The secretory response in permeabilized TG 1B3 cells at defined concentration of free calcium will be investigated.

Publications:

WoldeMussie, E. and Beaven, M.A.: Rat mast cell/basophil heterogeneity related to cell maturation. In _Mast Cell Differentiation and Heterogenity_, A.D. Befus, J. Bienenstock, A.D. Befus, and J.A. Denburg (Eds.), Raven Press, New York, 1986, 257-262.

WoldeMussie, E., Ali, H., Takuishi, T., Siraganian, R. and Beaven, M.A.: Identification of variants of basophilic leukemia (RBL-2H3) cells that have defective phosphoinositide responses to antigen and stimulants of GTP-regulatory proteins. _J. Immunol._, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00983-02 LCP

PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Mechanism of MPTP induced cell death

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

| | | | |
|---|---|---|---|
| P.I.: Anna Romany | Guest Researcher | LCP | NHLBI |
| Other Investigators: | | | |
| Y. Singh | Guest Researcher | LCP | NHLBI |
| G. Krishna | Chief, Section | LCP | NHLBI |

COOPERATING UNITS (if any)

Dr. Donald Bogdanski, Hypertension Endocrine Branch, NHLBI

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

| 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.)

Deprenyl, a specific monoamine oxidase-B inhibitor (MAO-B) has been shown to prevent MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced neuronal cell death and thus over coming MPTP induced Parkinson's disease. MPTP is converted to MPP+ (1-methyl-4-phenyl-pridinium), which is responsible for cell death. Last year we showed that deprenyl completely prevented MPTP induced cell death in cultured hepatocytes without any affect on MPP+-induced cell death. We have now investigated the structural requirement of deprenyl to induce this effect both in vivo and in vitro. Three structural analogs of deprenyl developed in Hungary in Dr. Knoll's Institute were used in the study. Of all the analogs, J508 was found to be most active in preventing hepatocytes cell death induced by MPTP. The inhibitory effect of all deprenyl analogs depended on the concentrations of MPTP employed. At concentration of MPTP below 0.5 mM, all three analogs completely prevented MPTP induced cell death. At 0.5 mM MPTP, however, the IC50 for inhibition cell death by the inhibitors were: deprenyl 11 μM, TZ650, 3 μM; U1424, 2 μM; and J508, 0.5 μM. These drugs were tested in vivo in pigmented mice against MPTP induced striatal dopamine depletion at doses of 1 and 3 mg/kg. All analogs of deprenyl completely prevented the depletion. For deprenyl, ED50 was about 0.3 mg/kg. It may be possible to develop deprenyl analogs that are more potent than deprenyl in humans in preventing development of Parkinson's disease.

Project Description:

Objectives: MPTP induces Parkinson's disease in humans and primates by a mechanism involving oxidation of the drug to MPP+ by MAO-B. A number of human studies are presently being pursued using inhibitors of MAO-B to prevent development of Parkinson's disease in susceptable individuals. We showed last year that hepatocytes in tissue culture could be successfully utilized for the study of MPTP induced cell death and a specific MAO-B inhibitor namely deprenyl prevented MPTP induced cell death. A number of MAO-B inhibitors have been developed in Dr. Knoll's Institute in Hungary. The main objective of this study is to screen these deprenyl analogs *in vitro* and compare it *in vivo* using a mouse model for MPTP induced striatal dopamine depletion as a biochemical model for Parkinson's disease.

Methods Employed: Rat liver cells were isolated as described in last years project report. Cell death was followed by enzyme and adenine nucleotide leakage. The *in vivo* studies black $C_{57}$ male mice weighing 20-25 g were employed. Mice were treated daily with 20 mg/kg MPTP for 7 days. MAO-B inhibitor was injected daily starting one day before MPTP injections at doses varying from 0.1 mg - 10 mg/kg. Dopamine and serotonin in the caudate nucleus along with parts of putamen-pallidum (striatam) were determined by HPLC using with an electrochemical detector.

The following drugs were used in the study:

1) $C_6H_5-CH_2-CH(CH_3)-N(CH_3)-CH_2-C\equiv CH$ — L-Deprenyl

2) $C_6H_5-CH_2-CH_2-N(CH_3)-CH_2-C\equiv CH$ — TZ 650

3) (indanyl) $N(CH_3) - CH_2 - C \equiv CH$ — J 508

4) (furyl) $CH_2 - CH(CH_3) - N(CH_3) - CH_2 - C \equiv CH$ — DL-U 1424

Major Findings: MPTP induced cell death was completely prevented by deprenyl indicating that MPP+ formation is essential for cell death. All the analogs tested also completely prevented cell death caused by concentrations of MPTP of less than 0.5 mM. At a MPTP concentration of 0.5 mM, however, these analogs inhibited the cell death in a concentration dependent manner. $IC_{50}$ of deprenyl was estimated to be 11 µM at 0.5 mM MPTP. J 508 was found to be more active with an $IC_{50}$ of 0.5 µM, where as TZ 650 and U 1424 had $IC_{50}$ values of 3 and 2 µM respectively. All deprenyl analogs inhibited the dopamine depletion by 20 mg/kg MPTP injected daily for 7 days. Since all analogs completely prevented MPTP effect in vivo at 1 mg/kg it has not been possible to estimate their relative potencies. The $ED_{50}$ for deprenyl was about 0.3 mg/kg.

Significance to Biomedical Research and to the Program of the Institute:
One of the major findings that there are specific MAO-B inhibitors which are more potent than deprenyl, which could utilized for the prevention of MPTP-induced damage. These inhibitors can also be developed for localization of MAO-B in different regions of the brain as well as in other organs such as liver, heart and lung in order to understand the role of MAO in physiologic as well as pathological conditions in heart, lung, and brain.

Proposed Course of Project: We plan to investigate the J 508 as a specific MAO-B inhibitor and the localization of MAO in various regions of the brain using radiolabeled drug. Since MAO-B appears to play an important role in MPTP as well as other drug-induced diseases, we propose to purify MAO-B to and prepare both monoclonal as well as polyclonal antibodies useful for cloning MAO-B gene.

Publications:

Rodrigues, M.M., Wilson, E.M., Wiggert, B., Krishna, G. and Chader, G.J.: Retinoblastoma - A clinical, immunohistochemical, and electron microscopic case report. Ophthalmology 93: 1010-1015, 1986.

Fletcher, R.T., Sanyal, S., Krishna, G., Aquirre, G. and Chader, G.J.: Genetic expression of cyclic GMP phosphodiesterase activity defines abnormal photoreceptor differentiation in neurological mutants of inherited retinal degeneration. J. Neurochemistry 46: 1240-1245, 1986.

Rodrigues, M., Hackett, J., Wiggert, B., Grey, I., Spiegel, A., Krishna, G., Stein, P. and Chader, G.: Immunoelectron microscopic localization of photoreceptor-specific markers in the monkey retina. Current Eye Research 6: 369-379, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00984-02-LCP

PERIOD COVERED
October 1, 1986 through September 30, 1987

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
A unique testosterone metabolite: 17β-hydroxy 4,6-androstadiene-3-one

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


| | | | | |
|---|---|---|---|---|
| P.I. | Kenneth Korzekwa | Staff Fellow | LCP | NHLBI |
| Others: | | | | |
| | James R. Gillette | Chief | LCP | NHLBI |
| | Henry Sasame | Chemist | | |


COOPERATING UNITS (*if any*)

Drs. Frank Gonzales and Mario Umeno (LMC, NCI)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.75 | 0.75 | |

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 space provided.*)


The purpose of this study is to probe the mechanism by which dexamethosone inducible cytochrome P-450 and P-450a convert testosterone to the metabolite 17β-hydroxy-4,6-androstadiene-3-one (Δ6T). Previous work in this laboratory, which showed that Δ6T formation paralleled 6-β-hydroxylation, suggested that the same P-450 isozyme(s) are involved and a dual hydrogen atom abstraction mechanism was proposed. An analogous metabolite of valproate has also been reported. The objective of this work is to measure the deuterium isotope effects associated with the desaturation mechanism. Selectively deuterated testosterone derivatives are being synthesized and will be subjected to metabolism by dexamethasone induced microsomes and preparations derived from COS cell cultures transfected with P-450a c-DNA.




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

Project Description:

Objectives: In previous work, this laboratory identified a new metabolite of testosterone, 17β-01-hydroxy-4,6-androstadiene-3-one (Δ6T) and found that its formation is dependent on the same cytochrome P-450 isozymes (dexamethasone inducible) responsible for 6β-hydroxylase activity (Drug Metabolism Disp. 14:559,1986). It was also shown that this metabolite does not result from the dehydration of 6 or 7 hydroxytestosterones. This suggested that a novel cytochrome P-450 desaturation mechanism is involved. Since aliphatic oxidation is known to occur via a hydrogen atom abstraction/recombination mechanism, it was proposed that the dehydro-metabolite is the result of a dual hydrogen atom abstraction mechanism. More recently, a cytochrome P-450 mediated metabolite of valproatic has also been reported (Science 235:890,1987). Also, the same testosterone metabolite (Δ6T) has been seen using a purified preparation of P-450a, a 7β-hydroxylating enzyme. Thus, it appears that desaturation pathways, while perhaps minor, may be common.

The objective of this project is to probe the mechanism of Δ6T formation by measuring the deuterium isotope effects associated with the desaturation step. Although isotope effects can often be "masked" by the many steps involved in an enzymatic sequence and therefore difficult to interpret, a dual hydrogen atom abstraction mechanism would be less susceptable to these effects. Since both hydroxylation and desatuation arise from the same intermediate (the radical produced by the first hydrogen atom abstraction), the magnitude of the observed isotope effect for the second hydrogen atom abstraction will primarily depend on the broaching ratios of the two apthways (see J.Amer.Chem.Soc.108:7074,1987). The ratio of hydroxylation to desaturation for testosterone metabolism by both enzymes is 10:1 suggesting that the intrinsic isotope effect for the second hydrogen atom abstraction will be observed.

Methods Employed: Substrates being synthesized include 6α,7α-$d_2$-testosterone, 6α,6β-$d_2$-testosterone, and 7β-$d_1$ testosterone. Deterium content and stereochemistry are being determined by GC-MS, proton and deuterium NMR. Enzyme preparations to be used include dexamethosone induced rat liver microsomes (as per reference above) and preparations from COS cells tranfected with P-450a c-DNA (in collaboration with Frank Gongelez). Quantitation of the metabolites will be performed by HPLC and the deuterium content of the metabolites will be determined by GC-MS.

Major Findings: Attempts to synthesize testosterone-(17β-$^2$H) have failed. We have synthesized testosterone-(6α,6β-$^2H_2$), and a mixture of testosterone-(6α, 7α-$^2H_2$) experiments with cytochrome P-450a and testosterone (6α, 6β-$^2H_2$) revealed isotope effects in the formation of Δ6T.

Publications:

Nagata, K., Liberto, D.J., Gillette, J.R. and Sasame, H.A.: An unusual metabolite of testosterone, 17β-hydroxy-4,6-androstadiene-3-one. Drug Metabolism Disp. 14: 559-565,1986.

Nagata, K., Matsunaga, T., Gillette, J., Gelboin, H.V. and Gonzalez, F.J.: Rat testosterone 7β-hydroxylase. Isolation, sequence and expression of cDNA and its developmental regulation and induction by 3-methylcholanthrene. J. Biol. Chem. 262: 2787-2793, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00985-02 LCP

PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Enzymatic reactions of purified cytochrome P-450 isozymes

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: | | | |
| James R. Gillette | Chief | LCP | NHLBI |


COOPERATING UNITS (*if any*)

Dr. Alan Buckpit, University of California, David, CA.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NIH, NHLBI-IR-LCP, Bethesda, Md. 20892

| 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.*)


Naphthalene is metabolized by cytochrome P-450 enzymes to two epoxides, namely, (1S), (2R)-naphthalene oxide and (1R), (2S)-naphthalene oxide. In the presence of glutathione and glutathione transferase mixtures of these epoxides are converted to three glutathione conjugates. Conjugates (1) and (3), which are (1S)-hydroxy-(2S)-glutathion-S-yl-1,2,-dihydronaphthalene and (1R)-glutathion-S-yl-(2S)-hydroxy-1,2-dihydronaphthalene are formed solely from (1S), (2R)-naphthalene oxide. Conjugate (2), which is (1R)-hydroxy-(2R)-glutathion-S-yl-1,2-dihydronaphthalene is formed solely from (1R), (2S)-naphthalene oxide (Buckpitt, personal communication. Two isozymes of cytochrome P-450 that metabolize naphthalene have been isoalted from mouse liver. Since reconstituted systems containing cytochrome $P\text{-}450_N$ and glutathione transferases convert naphthalene almost solely to conjugate 2, cytochrome $P\text{-}450_N$ must oxidize naphthalene almost solely to (1R), (2S)-naphthalene oxide. By contrast, systems containing cytochrome $P\text{-}450_S$ oxidize naphthalene to both epoxides but preferentially to (1S), (2R)-naphthalene oxide. Studies on the metabolism of naphthalene by microsome of mouse lung suggest that they contain predominantly cytochrome $P\text{-}450_N$ whereas those by microsomes from mouse liver, suggest that they contain both forms, but mainly cytochrome $P\text{-}450_S$. Since naphthalene causes pulmonary damage but not liver necrosis in mice, it may be that (1R), (2S)-naphthalene oxide is more toxic than (1S), (2R)-naphthalene oxide.




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

Project Description:

Objectives: Naphthalene causes pulmonary injury in Clara cells of mice but not in rats. Since naphthalene is metabolized to two different epoxides, it seems possible that the inter organ and inter species differences may be due to differences in either the intrinsic toxicities of these epoxides or the kinetics of their inactivation. The objective of this project is to differentiate between these possibilities.

Methods Employed: The HPLC method developed by Buckpitt and his associates for separating the three glutathione conjugates of the reactive metabolites of naphthalene was used.

Major Findings: Buckpitt and his associates have found that the 9,000 x g supernatant fraction of mouse liver converts naphthalene to three glutathione conjugates. In subsequent work they have found that the three conjugates were: 1) (1S)-hydroxy-(2S)-glutathion-S-yl-1,2-dihydronaphthalene. 2) (1R)-Hydroxy-(2R)-glutathion-S-yl-1,2-dihydronaphthalene. 3) (1-R)-glutathion-S-yl-(2R)-glutathion-S-yl-1,2-dihydronaphthalene. They have further proved that in the presence of a mixture of glutathione Transferase conjugates (1) and (3) are formed solely from (1S), (2R)-naphthalene oxide and conjugate (2) is formed solely from (1R), (2S)-naphthalene oxide.

Last year, we reported the isolation of two isozymes of cytochrome P-450, designated cytochrome P-$450_S$ and cytochrome P-$450_N$ and have produced polyclonal antibodies against each of them. Unfortunately both antibody preparations react with both isozymes, but both preparations inhibit the metabolism of naphthalene. Attempts to obtain specific antibodies from the preparations have thus far failed.

Nevertheless, we have found that the two purified isozymes differ markedly in their relative rates of formation of the glutathione conjugates of the naphthalene metabolites. Cytochrome P-$450_N$ formed the conjugates in relative amounts of 1:50:1, which suggests that about 96% of the naphthalene oxides was (1R), (2S)-naphthalene oxide. By contrast, cytochrome P-$450_S$ formed them in relative amounts of 1:1:1, which suggests that it forms both naphthalene oxides, but mostly (1S), (2R)-naphthalene oxide.

On the assumption that the naphthalene oxides are formed solely by these two isozymes of cytochrome P-450, it appears that there are marked interorgan differences in their relative distribution. With microsomes from mounse lung, the three conjugates were formed in relative amounts of 1:17.5:1.11, whereas with microsomes from mouse liver the conjugates were formed in relative amounts of 1:2.9:1.41. Since naphthalene causes cell necrosis in lung but not in liver, it seems possible that for unknown reasons, the (1R), (2S)-naphthalene oxide is more toxic than (1S), (2R)-naphthalene oxide.

Significance to Biomecial Research and to the Program of the Institute: It may be important to identify isozymes of glutathione transferase that catalyzes the formation of different stereoisometric metabolites and to study the route of detoxication.

Proposed Course of Project: 1) Attempt to isolate specific antibodies against cytochrome P-$450_M$ and cytochrome P-450s. 2) Attempt to study the kinetics of inactivation of the two naphthalene oxides by glutathione transferases.

Publications:

Sasame, H.A., Liberato, D.J. and Gillette, J.R.: The formation of glutathione conjugate derived from propranolol. Drug Metabolism and Disposition 15: 349-355, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00986-02 LCP

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Pharmacokinetic models for the study of reactive metabolites

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 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. 20892

| 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.)


This Laboratory has historically been involved in the study of tissue lesions mediated by the formation of chemically reactive metabolites of drug and other foreign compounds. A method has been developed to estimate in vivo the intrinsic clearances of enzymes that catalyze the formation of short-lived reactive metabolite. The basis for this method is that at low doses of the parent compound, the reactive metabolite preferentially reacts with endogenous substances such as glutathione (GSH). The depletion of GSH can be served as an index for the formation of the reactive metabolite. The assumptions made in this method are 1) the concentration of hepatic reactive metabolite reaches a steady state almost instantaneously, 2) the rate of the formation of reactive metabolite follows first order kinetics, and 3) the rate of the formation of the GSH conjugate follows second order kinetics. The animal model for this study was male hamsters. A marginally toxic dose of acetaminophen was used. The intrinsic clearance for the formation of chemically reactive metabolite was estimated from the rate of synthesis of hepatic GSH (calculated), the fraction of the dose of acetaminophen converted to GSH conjugate and reported values of hepatic blood flow. This intrinsic clearance for reactive metabolite formation was errified by measuring the rates of formation of the GSH conjugate in the 9000 x g supernatant fraction of hamster liver.




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

Project Description:

Objective: Many pharmacokinetic methods using compartment models have been used to evaluate the *in vivo* pharmacokinetics of long lived, and chemically stable metabolites. But there are few useful procedures for evaluating the *in vivo* pharmacokinetics of short-lived metabolites. The objective of this research is to developed simple procedures for estimating the organ clearance for the formation of a short-lived chemically reactive metabolite that initially is inactivated preferentially by reaction with endogenous GSH which becomes depleted as a consequence. Formation of the chemically reactive metabolite of acetaminophen in the liver of hamsters was used to demonstrate the usefullness of these procedures.

Methods Employed: Male Syrian hamsters were allowed access to food and water before the experiment. A dose of 225 mg/kg of acetaminophen was given sc at 8:00 AM. At predetermined intervals blood was drawn and animals sacrificed to obtain liver.

GSH assay. Reduced and oxidized hepatic GSH concentrations were both measured. Livers were homogenized in 9 volumes of 0.1 M phosphate buffer (pH 7). An equal volume of 4% sulfosalicyclic acid was added, and the mixture vortexed and centrifuged at 6000 x g for 10 min. To 0.5 ml of supernatant was added 4.5 ml of 0.1 mM dithionitrobenzoic acid in 0.1 M phosphate buffer (pH 8.0). The reduced GSH concentrations were determined spectrophotometrically by measuring the absorbancies of the lightly colored p-nitrothiophenol anion at 412 nm and relating them to standard curves. Oxidized GSH was reduced separately by GSH reductase and measured as above.

Serum acetaminophen: Blood was taken retro-orbitally at predetermined time intervals prior to the excision of the liver. Serum acetaminophen was determined by spectrophotometric measurement of the aminophenol produced by aryl acyl amidase hydrolysis of acetaminophen.

Unbound acetaminophen in plasma: Unbound acetaminophen was determined by dialysis of plasma in 0.96 M sodium potassium phosphate buffer (pH 7.0) for 9 to 12 hr.

Formation of glutathionyl acetaminophen in vitro. Hamsters were fasted overnight and injected ip with sodium pentobarbital (150 mg/kg) and heparin (14 USP units). The abdominal cavity was opened after loss of the righting reflex. The liver was perfused with 0.9% saline then homogenized with 3 times volume of 1.15% KCl, 20 mM Tris HCL (pH 7.4) and centrifuged for 20 min at 9,000 x g. The GSH conjugate was formed by incubating 0.125 g liver from the homogenate with 75 mM KCl, 20 mM in organic phosphate, 15 mM $MgCl_2$, a NADPH generating system consisting of 0.83 mM NADP. 20 ml glucose-6-phosphate, 4IU of glucose-6-phosphate dehydrogenase, 1 mM of GSH, and [$^3$H] acetaminophen. The ion mixtures incubate (3 ml) were shaken for 10 min at 37°C. Two volumes of ice cold acetone were added to stop the reaction. The precipitated incubates were refrigerated for 1 hr and centrifuged at 1,000 g for 20 min. The supernatant was filtered through a 0.45 μM Millipore filter and blown under a stream of $N_2$ to remove acetone and then lyophilized and redissolved in 0.25 ml glass distilled water.

HPLC identification of GSH conjugate. GSH conjugate was analyzed by a Waters HPLC system and a reverse phase Whatman Partisil 10 ODS-3 column. The chromatographic analysis was performed at room temperature using a mobile phase of 12.5% methanol/1% glacial acetic acid/86 5% water at a flow rate 1 ml/min. The injection volume was 5 µl. The eluate was collected and dissolved in 2 ml of methanol, 15 ml of Ready-Solve and counted for 10 min.

Simulations: Simulations were performed with MLAB or with an Apple IIe computer supported by the spreadsheet of Apple Works.

Major Findings: In previous years, we developed equations based on the total areas under the curves of acetaminophen and acetaminophen-induced loss of hepatic glutathione for estimating the fraction of the dose of acetaminophen that was converted to the reactive metabolite in liver, $F_H^{A,M}$, the clearance of formation of the reactive metabolite in liver $CL_H^{A,M}$ and the apparent intrinsic clearances of the hepatic enzymes that catalyze the formation of the metabolite $CL_{int,H}^{A,M}$ After incorporating the data obtained with hamsters dosed with acetmainophen into the equation, we calculated that: $F_H^{A,M} = 0.29$ and $CL_H^{A,M} = 0.297$ ml min$^{-1}$. Without knowing the values of the heaptic blood flow, it is not possible to estimate accurately the apparent intrinsic clearances of the enzymes that catalyze the formation of the chemically reactive metabolite within liver. Nevertheless, using estimates of hepatic blood flow obtained from the literature, we could calculate that the apparent intrinsic clearances should be between 0.58 and 0.67 ml min$^{-1}$. By contrast, calculation of the intrinsic clearance of the enzymes based on the rates of formation of glutathionyl acetaminophen by 9,000 x g supernatant fractions of hamster liver were 0.45 ml min$^{-1}$.

This year we also developed equations for estimating the values of $CL_H^{A,M}$ from data obtained solely during the initial depletion of glutathione. These equations are based on partial areas under the curves of acetaminophen and acetaminophen-induced loss of glutathione. They are potentially very useful because they permit us to detect nonlinearities in the data and to test the hypothesis that virtually all of the reactive metabolite is inactivated by combination with glutathione. Indeed, graphs of the in vivo data based on these equations revealed no evidence of the nonlinear kinetics predicted by the in vitro experiments, but did suggest that glutathione synthesis was maintained at constant rates during the depletion and repletion of glutathione.

Signifiance to Biomedical Research and Program of the Institute: It is known that most chemically reactive metabolites are formed by enzymes in the liver. The procedures developed in this research should be useful in studying kinetic parameters for virtually any short-lived chemically reactive metabolite that forms GSH conjugate. Similar equations may also be useful in studying the effects of short-lived reactive metabolites on other enzymes that are irreversibly inactivated but subsequently regenerated in target cells.

Proposed Course of Project: These procedures may be useful in distinguishing differences between species and strains of animals and lesions caused by different treatments which affect the formation of the short-lived reactive metabolite.

Publication:

Gillette, J.R.: Route, dose and species extrapolation. Natl. Research Council Publication, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00987-01 LCP

PERIOD COVERED
October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Studies on the active sites of cytochromes P-450

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

| | Name | Title | Lab | Institute |
|---|---|---|---|---|
| P.I. | Kenneth Korzekwa | Staff Fellow | LCP | NHLBI |
| Other: | James R. Gillette | Chief | LCP | NHLBI |

COOPERATING UNITS (if any)

Drs. Frank Gonzales and Morio Umeno (LMC, NCI)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.25 | 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.)

Cytochrome P-450a is a P-450 isozyme showing a high degree of regiospecificity for the 7α-hydroxylation of steroids. The steric and electronic binding properties responsible for this specificity will be probed by evaluating the metabolism of several steroid analogs or fragments. Models used in this study include aliphatic and unsaturated ring systems steroid fragments, and a potential heme-binding optically active steroid analog. The regioselectivity, chiral and prochiral selectivity, and kinetics of the metabolism of these substrates will be used to evaluate the geometric properties of the P-450a active site.



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

Project Description:

Objectives: Cytochrome P-450a is a monooxygenase isozyme showing unusual regiospecificity for the 7α-hydroxylation of some steroids. Since this position has no unusual electronic characteristics to increase its reactivity, this specificity is likely to be the result of the steric and electronic properties of the active site. In order to probe these properties, a series of testosterone analogs and fragments will be subjected to metabolism by cytochrome P-450 preparations. The regiospecificity and kinetic constants associated with the metabolism will be used to estimate the relative contributions of the steroidal geometrics to binding and metabolism. The substrates studies will conclude the following: 1) Trans-decalin (decahydronaphthalene). This substrate is a saturated bicyclic compound which can correspond to the A + B or B + C ring of the steroid structures. Along with the regiospecificities which can be measured, a more subtle effect can also be quantitated. While the trans decalin molecule is simple aliphatic compound, all of the carbon atoms are pro-chiral. Metabolism one side of the two ring surface (i.e. the "top") will result in a different stereoisomer than metabolism on the other side (i.e. "bottom"). Differences in the rates of formation for each isomer would suggest that the precise geometry of the flat aliphatic (α-face) of the steroid may be involved in substrate binding. 2) β-keto decalin. This substrate corresponds to the A + B rings of the 3-ketosteroid. Metabolism of the 7-position of this substrate would correspond to metabolism at 7-steroidal positions. Preferential metabolism of the 7α-position would be expected if the 3-keto group is important in substrate binding. 3) β-keto-1-decalene. This substrate also corresponds to the A + B steroid rings and includes the Δ4-double bond. The ene-one functionality would form a stronger hydrogen bond than the 3-keto substrate described above. 4) (S) and (R) 4,4a,5,6,7,8-hexahydro-4a-methyl-2(3H)-naphthalenone stereoisomers. The (S) isomer corresponds directly to the A/B rings of the Δ4-3-keto steroids ((i.e. keto hydroxyprogesterone, etc.) including the 19β-methyl group. The (R) isomer is analogous to the same rings with the α and β faces inverted (i.e. methyl group down). The steric and binding effects of the 19-methyl group can be probed with this substrate. 5) Δ6-androstene-17-βol-3-one. This substrate may be a suicide substrate for P-450a due to the isolated double bond at the 6-position. Analysis of the heme adducts will determine the position of substrate binding relative to the different porphyrin rings.

Methods Employed: The enzyme preparations used for these metbolic studies will be obtained from COS cells transfected with P-450a c-DNA. Substrate 1 and 4 are commercially available. Substrates 2 and 3 can be prepared by one and three standard reaction methods, respectively. Metabolites of (1) will be derivatized with a chiral agent, and the diastereroisomers separated and quantitated by HPLC. Compound (2) will be derivatized with a UV active compound for HPLC analysis. Determination of the structures of the metabolites will be determined by GC-MS.

Major Findings: Experimental portion of project has not yet been started.

Publication:

Gillette, J.R.: Problems and caveats in enzyme induction in man. Taylor and Francis, Ltd., in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00988-01 LCP

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Metabolic basis for enflurane hepatotoxicity

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

| | | | |
|---|---|---|---|
| P.I. David Christ | Guest Researcher | LCP | NHLBI |
| Others: | | | |
| Lance R. Pohl | Section Chief | LCP | NHLBI |
| Hiroko Satoh | Vist. Assoc. | LCP | NHLBI |
| J. Gerald Kenna | Guest Researcher | LCP | NHLBI |

COOPERATING UNITS *(if any)*

William Kammerer, Anesthesiology Section, Clinical Center, NIH.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Pharmacological Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.25 | 1.25 | |

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.)*

Case reports of idiosyncratic enflurane hepatitis and an apparent cross-sensitization between halothane and enflurane exposure have suggested to us that the oxidative metabolism of enflurane to a reactive acylating intermediate might produce covalently bound protein adducts which act as antigens, similar to those implicated in the genesis of halothane hepatitis. Use of immunoblotting and enzyme linked immunosorbent assay techniques revealed that several microsomal protein adducts that react with an anti-trifluoroacetyl (TFA) hapten antibody were formed in rat liver after halothane, enflurane, or isoflurane administration. The relative extents of adduct formation were halothane >> enflurane >> isoflurane and correlated with the relative rates of metabolism of these drugs. Moreover, antibodies found in the serum of patients with fulminant hepatic necrosis induced by halothane, recognized these adducts. These studies indicate that a common molecular mechanism involving potentially immunogenic, covalently bound metabolites may be responsible for the idiosyncratic hepatitis seen after enflurane in patients sensitized to halothane. Moreover, they also suggest that the relatively safe inhalation anesthetic, enflurane, probably should not be administered to patients that have been sensitized previously to halothane or enflurane.



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

Project Description:

Objectives: The intent of this investigation is to determine the molecular basis of enflurane-induced hepatotoxicity and the basis of the apparent cross-sensitization with halothane.

Methods Employed: Male Sprague-Dawley rats were treated with single doses of halothane, enflurane, isoflurane or sesame oil vehicle, then killed 15 hr later and hepatic microsomes prepared. In a separate experiment, rats received 3 doses of enflurane or isoflurane, and were sacrificed 15 hr later and microsomes prepared. The microsomal fractions were tested for the presence of covalently-bound protein adducts by immunoblotting and enzyme linked immunosorbent assay (ELISA) techniques using a specific anti-TFA hapten antibody, and sera from patients with severe halothane-induced hepatic necrosis. Liver biopsy samples have been obtained from patients after initial induction with a barbiturate, narcotic, and nitrous oxide, and then after clinical anesthesia with enflurane, isoflurane or halothane for 2 hr. Microsomal fractions of these samples have been prepared, and preliminary immunoblotting experiments using the anti-TFA antibody and serum from halothane hepatitis patients are in progress.

Major Findings: Immunoblotting and ELSIA techniques using anti-TFA hapten antibodies or sera from patients with severe halothane-induced hepatic necrosis clearly demonstrated the formation of hepatic microsomal protein-adducts (Mr approximately 54, 59, 76, and 100 K) after enflurane, halothane administration to rats. Small amounts of adducts were also formed after isofluorane, as measured by immunoblotting. Formation of these adducts was more pronounced after multiple doses of the anesthetics. The magnitude of adduct formation, halothane >> enflurane >> isoflurane, directly correlates with the relative rates of metabolism of these drugs. Therefore, although the chemically reactive intemediate produced by the oxidative metabolism of enflurane is chemically different from that produced by halothane, it is still structurally similar enough for the haptens to cross-react with both the anti-TFA hapten antibody and the antisera from patients with severe halothane-induced hepatic necrosis.

Significance to Biomedical Research and Program of the Institute: Each year, millions of surgical procedures are performed world-wide which utilize halothane, enflurane or isoflurane anesthesia. Although the number of patients developing idiosyncratic hepatic necrosis after exposure to these drugs is relatively small, the severity of this syndrome and its potentially fatal outcome emphasize the importance of understanding the biological mechanisms responsible for this drug-induced tissue damage. The present studies indicate that a common molecular mechanism involving potentially immunogenic, covalently bound metabolites, may be responsible for both the idiosyncratic hepatitis seen after halothane or enflurane treatments and the apparent cross-sensitization between halothane exposure and the development of hepatic necrosis after subsequent enflurane anesthesia. These findings may lead to the design of safer inhalation anesthetic drugs which are not susceptible to metabolic activation and the production of potentially immunogenic protein adducts. Furthermore, these

results and techniques should be applicable to the design of experiments examining the possible role of the immune system in other idiosyncratic, drug-induced toxicities.

Proposed Course of Project: Future studies will initially focus on the immunochemical identification of protein-acyl metabolite conjugates of halothane, enflurane, and isoflurane in human liver, in order to confirm the medical relevance of the animal studies. The next step will be to investigate how the antigen presenting cells of the immune system in the liver, such as the Kupffer cells, process the protein-acyl conjugates in preparation for their presentation to and activation of specific helper T lymphocytes.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00989-01 LCP

PERIOD COVERED
October 1, 1986 through September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Characterization of a novel 59 kDa form of cytochrome P-450

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:

| Name | Title | Lab | Institute |
|---|---|---|---|
| Lance R. Pohl | Section Chief | LCP | NHLBI |
| David D. Christ | Guest Researcher | LCP | NHLBI |
| J. Gerald Kenna | Guest Researcher | LCP | NHLBI |
| John W. George | Chemist | LCP | NHLBI |


COOPERATING UNITS (if any)
David Kupfer, Worcester Foundation for Experimental Biology, Shrewsbury, MA:
Frank J. Gonzales, Laboratory of Molecular Carcinogenesis, NCI.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Pharmacological Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 1.75 | 1.25 | 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.)


We previously reported that one major trifluoroacetylated (TFA) protein (TFA-59 kDa) was detected in rat liver microsomes 12 hr after halothane (H) treatment. Its CO-reduced difference spectrum (450.6 nm) was consistent with it being cytochrome P-450. We have now further characterized this protein and indeed it appears to be a constitutive form of cytochrome P-450. When rat liver microsomes from untreated rats were incubated with H and NADPH, the 59 kDa protein became labeled with the TFA moiety, suggesting that it had metabolized H to the reactive acylating species, trifluoroacetyl halide. Antiserum prepared against TFA-59 kDa reacted with proteins in the microsomes of liver (59 and 60 kDa), lung (59 kDa), adrenal (59 kDa), testes (59 kDa), and fat (59 kDa) of untreated rats and in the liver of a human (59 kDa). A 59-kDa cytochrome P-450 purified rabbit liver (JBC 260:2027, 1985), which hydroxylates regioselectively prostaglandins in the omega position, cross-reacted with the anti-TFA 59 kDa antiserum and also appeared to become labeled with the TFA group, when halothane was administered to rabbits. It appears that the 59 kDa protein is a previously uncharacterized form of rat cytochrome P-450, which is immunochemically related to rabbit and human liver proteins and several other proteins in various tissues of the body.




PHS 6040 (Rev. 1/84)

GPO 914-918

Project No. Z01 HL 00989-01 LCP

Project Description:

Objectives: The intent of this investigation is to determine the physiological function and the regulation of the 59 kDa form of cytochrome P-450.

Method Employed: Rat liver microsomes from untreated rats were incubated with NADPH and H and the resulting TFA proteins were analyzed by Western blot analysis with anti-TFA antiserum as previously described. Anti-TFA-59 kDa was raised in rabbits and used to identify immunochemically related proteins in various tissue samples by Western blot analysis.

Major Findings: When rat liver microsomes from untreated rats were incubated with H and NADPH, the 59 kDA protein became labeled with the TFA moiety, suggesting that it had metabolized H to the reactive acylating species, trifluoroacetyl halide. Antiserum prepared against TFA-59 kDa reacted with proteins in the microsomes of liver (59 and 60 kDa), kidney (60 kDa), lung (59 kDa), adrenal (59 kDa), testes (59 kDa) and adipose tissue (59 kDa) of untreated rats and in the liver of a human (59 kDa). A 59-kDa cytochrome P-450 purified from rabbit liver (JBC 260,2027,1985), which hydroxylates regioselectively prostaglandins in the omega position, cross-reacted with the anti-TFA-59 kDa antiserum and also appeared to become labeled with the TFA group, when halothane was administered to rabbits. The anti-TFA-59 antiserum, however, did not block the activity of the rabbit enzyme.

Significance to Biomedical Research and Program of the Institute: The 59 kDa protein appears to a novel constitutive form of cytochrome that is present in most tissues of the body. It likely has an important physiological role in the synthesis and or metabolism of endogenous compounds such as steroids, fatty acids (arachidonic acid), prostaglandins, or leukotrienes. Therefore a thorough understanding of the function and regulation of this enzyme should enhance our knowledge of cellular physiology and pathology.

Proposed Course of Project: We intend to do the following: 1) Since the 59 kDa protein is present in tissues at low levels, it is difficult to isolate enough of the enzyme by conventional chromatographic techniques to determine its enzymatic function. To circumvent this problem, we intend to clone the cDNA of the protein, in collaboration with Dr. Frank Gonzales, from a lambda gt 11 expression cDNA library of normal rat liver microsomes tissue using our anti-TFA-59 kDA antiserum for screening of the library. We shall attempt to express the cDNA in yeast in order to isolate sufficient quantities of the enzyme to characterize its physiological function. 2) The subcellular tissue localization of the protein will be determined by light and electron microscopic immunochemistry, in order to learn more about the function of the enzyme. 3) The regulation of the enzyme will also be studied in order to characterize more completely its physiological function and potential role in pathological conditions.

Publications:

Satoh, H., Gillette, J.R., Takemura, T., Ferrans, V.J., Jelenich, J.G., Kenna, J.G., Neuberger, J. and Pohl, L.R. Investigation of the immunological basis of halothane-induced hepatotoxicity in Biological Reactive Intermediates III (Snyder, Jollow, Kocsis and Nelson, Eds.) Plenum Press, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00990-01

PERIOD COVERED

October 1, 1986 to September 30, 1987

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

Biochemical mechanisms of mast cell degranulation: Studies with disrupted cells

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


| | | | | |
|---|---|---|---|---|
| P.I.: | Hydar Ali | Visiting Fellow | LCP | NHLBI |
| Others: | | | | |
| | Michael A. Beaven | Deputy Chief | LCP | NHLBI |
| | J.R. Cunha-Melo | Visiting Associate | LCP | NHLBI |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

| 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.)


Antigen-mediated exocytosis in RBL-2H3 cells is associated with substantial hydrolysis of membrane inositol phospholipids and an elevation in concentration of of cytosol Ca(2+) ([Ca2+]). However, the relationship between production the intracellular Ca(2+) releasing agent, inositol 1,4,5-trisphosphate, and the increase in [Ca(2+)] has been established from studies with intact cells. It is shown that cells labeled with [3H]myoinositol and permeabilized with streptolysin O do release [3H]inositol 1,4,5-trisphosphate upon stimulation with antigen or guanosine 5'-O-(3-thiotriphosphate) (GTP $\gamma$ S) at low (<100 nM) concentrations of free Ca(2+). The response, however, is amplified by increasing free Ca(2+) to 1 μM. As cytosol proteins and presumably inositol 1,4,5-trisphosphate 3'kinase leak from the permeabilized cells, conversion of the trisphosphate to inositol 1,3,4,5-tetrakisphosphate is reduced. As a consequence, [3H]inositol 1,4,5-trisphosphate accumulates in greater amounts than is the case in intact cells. Nevertheless, [3H]inositol 1,4-bisphosphate is the major product in permeabilized cells even when [3H]inositol 1,4,5-trisphosphate is trapped by the presence of excess (500 μM) unlabeled inositol 1,4,5-trisphosphate and the phosphatase inhibitor 2,3-bisphosphoglycerate. It would appear that both phosphatidylinositol 4-monophosphate and the 4,5-bisphosphate are substrates for the activated phospholipase C and that these two lipids have rapid turnover times (< 120 sec) ) at maximal rates of stimulation.




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

Project Description:

Objectives: RBL-2H3 cells can be stimulated to secrete histamine by aggregation of IgE-receptors, either directly by addition of chemically cross-linked oligomers of IgE or indirectly by antigen in cells primed with the appropriate antigen-specific IgE. Such stimulation causes substantial hydrolysis of membrane inositol phospholipids and an increase in cytosolic concentrations of $Ca^{2+}$-reactions that are dependent on the size of receptor clusters and number of receptors aggregated. As discussed in another report (Z01 HL 00937-04 LCP), analysis of the inositol phosphates by HPLC techniques indicated an early production of inositol polyphosphates and a complex pattern of degradation of the polyphosphates in stimulated RBL-2H3 cells. The studies did not establish, however, that the $Ca^{2+}$-mobilizing agent, inositol 1,4,5-trisphosphate was the primary product of hydrolysis or that it mediates the generation of all the inositol phosphates.

To simplify the kinetic picture in this project we have resorted to permeabilized dialyzed cells which allowed us to use $Ca^{2+}$-buffers with defined concentrations of free $Ca^{2+}$ that pertain to those within the intact cell. Also radiolabeled products could be trapped by addition of excess unlabeled product.

Methods Employed: All procedures were performed as described in previous reports. The permeabilization of cells was achieved by use of streptolysin O as described by Howell and Gomperts (BBA 927:173,1987).

Major Findings: 1) Antigen and GTP$\gamma$S mediate PI hydrolysis in permeabilized RBL-2H3 cells. Substantial release of [$^3$H]inositol phosphates was induced in permeabilized cells by antigen ($DNP_{24}BSA$). Unexpectedly even greater responses could be elicited with permeabilized cells than with intact cells, especially with high concentrations of antigen. The response in permeabilized cells was dependent on the presence of ATP and was modulated by the nature of anions in the buffer. Permeabilized cells could be stimulated even when the free $Ca^{2+}$ was virtually zero. Increasing the free concentration of $Ca^{2+}$ to 1 μM increased the release of [$^3$H]inositol phosphates in response to low concentrations of antigen or GTP $\gamma$S. Such amplification was not observed with concentrations of antigen that caused a maximal release of [$^3$H]inositol phosphates.

An attractive feature of the permeabilized cells was that extensive washing of cells resulted in loss of protein kinase C and loss of responsiveness to antigen and GTP$\gamma$S. Also desensitization of intact cells to high concentrations of antigen was not observed with permeabilized cells.

2) Production of [$^3$H]inositol 1,4,5-trisphosphate and [$^3$H]inositol 1,4-bisphosphate in dialyzed RBL-2H3 cells. Studies in which labeled products were trapped by addition of excess unlabeled metabolite and phosphatase inhibitors indicated that inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate were the two primary products of hydrolysis of the inositol phospholipids. The reaction occurred at concentrations of free $Ca^{2+}$ that were biologically relevant to those in the intact RBL-2H3 cells (i.e. 0.1 μM and about 1 μM).

The production of [$^3$H]inositol 1,4-bisphosphate, as well as inositol 1,4,5-trisphosphate has the interesting implication that the two phosphorylated derivatives of phosphatidylinositol are substrates for phospholipase C in the permeabilized RBL-2H3 cells. The estimated rate of production of inositol 1,4,5-trisphosphate was (mass measurements were performed by J. Moyer, NCI, DCT, LBC) 3.5 μmoles/L/min.

3) Formation of inositol 1,3,4,5-tetrakisphosphate from inositol 1,4,5-trisphosphate. The appearance of [$^3$H]inositol tetrakisphosphate followed that of [$^3$H]inositol 1,4,5-trisphosphate, a sequence that was not clearly demonstrated in studies with intact RBL-2H3 cells. Interestingly the rate of accumulation of the tetrakisphosphate was enhanced by increasing the concentration of free $Ca^{2+}$ to $10^{-6}$M, a possible indication that any residual 3'-kinase activity in the permeabilized cells could be modulated by $Ca^{2+}$. As both inositol 1,3,4,5-tetrakisphosphate and its dephosphorylated product, inositol 1,3,4-trisphosphate, were increased these two products were derived from [$^3$H]inositol 1,4,5-trisphosphate.

4) Degradation of the inositol polyphosphates. The initial step was shown to be the removal of the 5'-phosphate from inositol 1,4,5-trisphosphate and from inositol 1,3,4,5-tetrakisphosphate by a 5'-phosphomonoesterase activity which was present in the membrane fraction of RBL-2H3 cells.

The final steps in the sequence of dephosphorylation, however, were impaired in permeabilized cells. There was little or no accumulation of [$^3$H]inositol monophosphate and [$^3$H]inositol. Loss of enzymes that catalyze the degradation of inositol bisphosphate and inositol monophosphate was possible as these enzyme activities are located exclusively in the cytosol of RBL-2H3 cells.

Significance to Biomedical Resarch and to the Program of the Institute. The studies begin to provide a detailed understanding of the signalling mechanism at a molecular level. During the course of this project it has been shown that (1) permeabilization of RBL-2H3 cells with streptolysin O leads to GTP S and antigen mediated PI hydrolysis at low (< 100 nM) concentrations of free $Ca^{2+}$; (2) the response is amplified by increasing the free $Ca^{2+}$ concentraion to 1 μM; (3) conversion of inositol 1,4,5-trisphosphate to inositol 1,3,4,5-tetrakisphosphate is reduced; (4) [$^3$H]inositol bisphosphate, however, predominates over that of inositol 1,4,5-trisphosphate; and (5) it appears that both phosphatidylinositol 4-monophosphate and the 4-5 bisphosphates are substrates for activated PLC.

Proposed Course of Project: Future studies will focus on the apparent loss of down regulation of receptor-mediated signals and on the loss of soluble factor(s) in permeabilized cells that are critical for signal generation. We will determine whether or not down regulation and signal generation can be restored by addition of purified protein kinase C, phospholipase C (both are available to us) or ras proteins. If so, the biochemical interaction of these proteins with plasma membrane components will be studied in detail.

Publications:

Maeyama, K., Hohman, R.J., Metzger, H. and Beaven, M.A.: Quantitative relationships between aggregation of IgE-receptors, generation of intracellular signals and histmaine secretion in rat basophilic leukemia (2H3) cells. Enhanced responses with heavy water. J. Biol. Chem. 261: 2583-2592, 1986.

Beaven, M.A.: Beyond the receptor: How stimulatory signals are transmitted in the cell; in Development of Drugs and Modern Medicines (Gorrod, J., Gibson, G.G. and Mitchard, M. (eds.). Ellis Horwood Publishers, Chichester, U.K. pp107-116, 1986.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00991-01 LCP

PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Signal generation and secretion of mediators 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 | | |


COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 0.7 | 0.7 | 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.)


Ionophore-induced secretion of histamine from rat basophil leukemic (RBL-2H3) cells correlated with the hydrolysis of inositol phospholipids but not with the increase in cytosol Ca(2+) ([Ca(2+)]) although increased [Ca(2+)]) was obligatory for secretion (J. Biol Chem. 262:414,1987). The same pattern was observed for release of arachidonic acid. Moreover, the release of both histamine and arachidonic acid was enhanced by activators of protein kinase C and in this circumstance the release was correlated with the increase in [Ca(2+)]). The extent of release of histamine and arachidonic acid to ionophore alone, therefore, may be limited by the extent of activation of protein kinase C rather than by the extent of increase in [Ca(2+)]). The subsequent mechanisms of release may well differ: the release of arachidonic acid was 4 to 5 times greater with optimal concentrations of A23187 than with optimal concentrations of antigen in the absence or presence of activators of protein kinase C. This was not true for release of histamine for which both ionophore and an activator of protein kinase C were required to match the release induced by optimal concentrations of antigen alone.




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

Project Description:

Objectives: Cultured rat basophilic leukemia (2H3) cells can be stimulated to secrete histamine either by aggregation of plasma membrane IgE-receptors with antigen when cells are primed with the antigen-specific IgE or by high concentrations (>200 nM) of ionophore A23187. Both forms of stimulation results in hydrolysis of membrane inositol phospholipids and an increase in cytosolic $Ca^{2+}$ concentrations (calcium signal). Last year we reported that the calcium signal stimulated by A23187 or antigen is dependent on intracellular ATP and that depletion of cellular ATP blocks hydrolysis of inositol lipids and secretion. Because antigen stimulation is associated with partial depolarization of the plasma membrane of 2H3 cells (Kanner and Metzger, PNAS 89:5744, 1983) and the calcium signal and secretion are blocked when these cells are depolarized by exposure to high $K^+$-containing medium (Mohr and Fewtrell J. Cell Biol. 104:783, 1987), we performed experiments to study whether ATP-depletion caused membrane depolarization of the 2H3 cells.

Another event observed upon stimulation of 2H3 cells is the release of arachidonic acid and its metabolites. However, the relationship, if any, of the stimulatory signals involved in the release of histamine and arachidonic acid is not known. As our previous studies have shown that A23187-induced secretion is potentiated by the activators of protein kinase C, 12-O-tetradecanoylphorbol 13-acetate (TPA) or Oleoyl-2-acetyl-rac-glycerol (OAg), we have studied the effects of TPA on the antigen- and A23187-stimulated release of arachidonic acid.

Methods Employed: Studies were conducted with RBL-2H3 cells in both suspension and monolayer culture. The inositol phospholipid pools were labeled by incubation of cells with [$^3$H]myoinositol overnight and then primed with monoclonal IgE. Accumulation of [$^3$H]inositol phosphates, in the presence of 10 mM $Li^+$, was determined as described in last years report (Z01 HL 00974-02 LCP). Cytosol calcium concentrations were monitored in quin 2 loaded cells. Histamine was assayed by radioenzymatic assay (J. Biol. Chem. 259,7129 and 7137,1984). 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.

Changes in the state of polarization of the plasma membrane were assessed by the distribution of the radiolabeled permeant cation, [$^{14}$C] (tetraphenylphosphonium bromide ($Ph_4P^+$) across the membrane. Where indicated glucose was omitted and antimycin A was added to the same solution. Cultures were labeled by overnight incubation with [$^{14}$C]arachidonic acid incubation at 37°C in an atmosphere of 95% air-5% $CO_2$. The medium was removed and the cells were washed twice with a buffered $Li^+$-containing salt solution (see last year's report). A solution of the same composition (180 µl) was added. The cultures were then stimulated with $DNP_{24}$ BSA (100 ng/ml) or an A23187 solution (25-1000 nM) in the presence or absence of TPA. Medium was removed and centrifuged (Beckman Microfuge B, 4°C, 90 sec) for assay of radioactivity and histamine. To assay for cellular $^{14}$C, the cells were extracted with a mixture (950 µl) of water-chloroform-methanol methanol as previously described for the isolation of inositol phospholipids. Release of $^{14}$C label was expressed as a percentage of total $^{14}$C in cellular lipids in unstimulated cultures.

Major Findings: Effects of depletion of cellular ATP on the state of membrane polarization of 2H3 cells: Equilibration of [$^{14}C$]$Ph_4P^+$ between medium and cells was achieved by 30 min when approximately 15% of the label had accumulated in the cells. The addition of $DNP_{24}BSA$ caused a 30% decrease in intracellular $^{14}C$ label, which is consistent with the reported partial depolarization of 2H3 cells upon stimulation with antigen.

Total cellular ATP content of cells after treatment with 2 and 10 mM antimycin were reduced by 52 $\pm$ 15% and > 95%, respectively, when compared with untreated cells. The same concentrations of antimycin A did not cause membrane depolarization in 2H3 cells at any of the time points studied. In experiments in which both [$^{14}C$]$Ph_4P^+$ antimycin A were added simultaneously, there was also no decrease in the extent of accumulation the permant cation. When cells were depolarized by exposure to the buffered solution in which NaCl was replaced by 124 mM KCl, the accumulation of the labeled probe was reduced by 77 $\pm$ 2%. Subsequent addition of $DNP_{24}BSA$ did not cause redistribution of the permeant cation. The data implied that the effects of ATP-depletion on stimulatory and secretory responses of RBL-2H3 cells were not attributable to depolarization of the plasma membrane.

A23187-stimulated release of [$^{14}C$]arachidonic acid: The ionophore A23187 caused concentration-dependent release of both [$^{14}C$]arachidonic acid and histamine. For example, at 200 nM A23187, the net release of [$^{14}C$]arachidonic acid and histamine was 6.3 $\pm$ 0.5% and 24.5 $\pm$ 1.9%, respectively; at 1000 nM A23187, the net release was 18.3 $\pm$ 0.7% and 61 $\pm$ 2%, respectively. Both responses were highly correlated with a $Ca^{2+}$-dependent stimulation of hydrolysis of inositol phospholipids (see last year's report). As lower concentrations of ionophore still elicited substantial increases in cytosolic $Ca^{2+}$, the increase alone was an insufficient-stimulus for secretion. Omission of external $Ca^{2+}$ ions blocked the rise in cytosolic calcium, the release of $^{14}C$ labeled arachidonic acid (i.e. < 0.5% release) and secretion of histamine (i.e. < 2% release) in response to A23187.

Comparison of the effects of phorbol ester on antigen- and A23187-responses in 2H3 cells. Short-term (30 min) treatment with TPA (50 nM) markedly enhanced the release of $^{14}C$ labeled arachidonic acid (from 4 $\pm$ 1.2% to 8.6 $\pm$ 0.7%) and histamine (from 9 $\pm$ 2.5% to 55.8 $\pm$ 2.2%) induced by 200 nM A23187. The hydrolysis of inositol phospholipids, on the other hand, was modestly suppressed by 50 nM TPA (e.g. a 3.7 $\pm$ 0.5% versus 2.6 $\pm$ 0.3 net release of inositol phosphates with 200 nM A23187).

In contrast, at optimal concentrations of antigen (10 ng/ml $DNP_{24}$ BSA) the phorbol ester caused only a small enhancement of release of [$^{14}C$]arachidonic acid and histamine. However, the treatment with TPA dramatically suppressed hydrolysis of inositol phospholipids (21.4 $\pm$ 1.4% versus 3.8 $\pm$ 0.2% release of inositol phosphates). By itself antigen was a more potent stimulant of histamine release (55 $\pm$ 3% release) and a less potent stimulant of [$^{14}C$]arachidonate release (3 $\pm$ 0.5%) than A23187.

Significance to Biomedical Research and to the Program of the Institute. Our studies have shown that ionophore-induced hydrolysis of inositol phospholipids, secretion of histamine and release of [$^{14}$C]arachidonic acid were highly correlated. All three responses, however, require extracellular calcium. Activation of protein kinase C with phorbol ester potentiates release of both substances whereas the putative stimulatory event, i.e. hydrolysis of inositol phospholipids, is suppressed. The release of the two mediators appears to share similar but not entirely identical mechanisms of release as indicated by the differences in the effects of A23187 and antigen. That protein kinase C has an integral role in the regulation of release of both substances provides the prospect of an additional way of therapeutic intervention in the treatment of allergic reactions.

Proposed Course of Project: The role of protein kinase C in the release of histamine and arachidonic acid will be explored further by use of inhibitors of protein kinase C and by depleting cells of protein kinase C by prolonged treatment with phorbol ester (unpublished data). In addition the possible modulation of histamine release by metabolites of arachidonic acid (e.g. $PGE_1$) through activation of adenylate cyclase will be investigated. This possibility has not been systemically studied in our previous work.

Publications:

Lo, T.N., Saul, W.F., and Beaven, M.A.: The actions of $Ca^{2+}$ ionophore on rat basophilic 2H3 cells are dependent on cellular ATP and hydrolysis of inositol phospholipids: a comparison with antigen stimulation. J. Biol. Chem. 262: 4141-4145, 1987.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00992-01 LCP

PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Mechanism of stimulatory, secretary and toxic responses of 2H3 cells to cardiotoxin

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


PI: Theresa N. Lo — Research Chemist — LCP — NHLBI

Other: Michael A. Beaven — Deputy Chief — LCP — NHLBI


COOPERATING UNITS *(if any)*

Dr. C.S. Lo, Uniformed Service University of The Health Science, Bethesda, Md.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
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| 0.6 | 0.6 | |

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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.)*


Cultured rat basophilic leukemia (RBL-2H3) cells previously labeled with myo-[2-3H]inositol for 24 hr showed an appreciable release of [3H] inositol phosphates and a reciprocal decline in [3H]inositol phospholipids upon exposure to cobra cardiotoxin (Naja naja kaauthia). The most significant effect was a selective increase in the amount of 3H-label in the phosphotidyl inositol monophosphate (PIP) pool. The extent of increase was dependent on concentration and time of exposure to cardiotoxin. The kinetics of redistribution of label within the various inositol lipid and inositol phosphate pools suggested that a major action was the stimulation of phosphatidylinositol kinase activity which converts phosphatidylinositol (PI) to PIP. This action was confirmed by assay of the enzyme activity in membrane preparations. Cardiotoxin also caused a time- and concentration-dependent secretion of histamine and release of lactate dehydrogenase (LDH) from the 2H3 cells. The release of histamine but not LDH was totally dependent on external calcium. The results suggest that cardiotoxin-stimulated release of histamine from 2H3 cells is through a Ca(2+)-dependent noncytotoxic process.




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

Project Description:

Objectives: Cobra venom contains membrane-active cardiotoxins that comprise basic polypeptides of about 60 amino acid units. The effects of cardiotoxins may result from alteration of the biological and physical properties of the plasma membrane of target cells. The mechanisms proposed for their actions include: 1) penetration and disruption of plasma membranes, 2) inhibition of membrane NaK-ATPase, 3) displacement of membrane $Ca^{2+}$, and 4) formation of pores in the membrane with a resultant increase in membrane permeability to ions such as $Na^+$ and $Ca^{2+}$. Our earlier studies showed that the toxin at micromolar concentrations induced increased levels of PIP, hydrolysis of membrane inositol phospholipids, and leakage of LDH in canine kidney (MDCK) cells and rat basophil 2H3 cells. We have explored in more detail the effect of toxin on the phosphoinositide - breakdown and the consequence of such an effect on cell function. As functional responses can be readily measured in 2H3 cells (i.e. histamine release) the 2H3 cell has been the primary model for study.

Methods Employed: Measurement of hydrolysis of [$^3$H]inositol phospholipids: The protocols for labeling of 2H3 cells with myo[2-$^3$H]inositol and quantification of the water-soluble inositol phosphates are described in Z01 HL 00974-03-LCP. Separation of the chloroform-soluble radiolabeled inositol phospholipids was performed by chromatography on thin layer Silica gel 60 $F_{254}$ aluminum foil plates (E. Merck, Darmstadt, West Germany) that had been treated previously with a solution of EDTA, 2 mM and potassium oxalate, 1% in $H_2O$. Unlabeled standards (25 µg of each inositol phospholipid: Sigma Chemical Corp.) were added to all chloroform extracts. The extracts were then evaporated to dryness under nitrogen. The residues were dissolved in a chloroform/methanol (2:1) mixture and applied to the plates. The chromatograms were developed with chloroform/methanol/4 N $NH_4OH$ (9:7:2 parts by volume). The location of the phospholipids was determined by exposing the plate to iodine vapor. The gel segments that contained the individual phospholipids were scraped from the plate and assayed for radioactivity.

PI kinase activity: The plasma fractions 15-83 µg protein in a 10 µl vol) were added to 100 µl of warm (30°C) solution that contained 50 mM Tris, 20 mM $MgCl_2$, 1 mM EDTA, 1.4 µCi (-$^{32}$P) ATP and 1 mM Tris-ATP (final concentration). After 10 min of incubation (30°C), the reaction was terminated and the inositol phospholipids were extracted by the addition of 75 µl of chloroform/methanol/4N HCl (10:20:2.2, v/v/v). The inositol phospholipids were analyzed as described above.

Release of LDH and histamine: Twenty microliter of the buffered salt solution cardiotoxin or antigen (aggregated ovalbumin in cells primed with ovalbumin - specific IgE) was added to the monolayer cells (200 µl) at 37°C. At the end of the incubation period, the medium was centrifuged (Beckman Microfuge, 4°C, 90 sec). A portion of the supernatant fluid was analyzed for LDH; another portion was assayed for histamine. The activity of LDH in medium and cell lysates was analyzed by the Sigma No. 226 UV assay kit. For total intracellular LDH activity, cells were lysed with 0.1% Triton X-100. Histamine was determined by radioenzyme assay as described in previous reports.

Major Findings: Effects of cardiotoxin on levels of individual inositol phospholipids in 2H3 cells: An isotopic equilibrium of the $^3H$ label was reached after 24 hr incubation (37°C) of the cells with [$^3H$]inositol. The distribution of the $^3H$ label among the inositol lipids were 87.4 $\pm$ 1.6% for PI, 3.9 $\pm$ 0.2% for PIP and 2.0 $\pm$ 0.7% for $PIP_2$ (n=5).

Upon addition of cardiotoxin (50 µg/ml) there was a substantial accumulation of the $^3H$ label in the cellular PIP pool (from 5% to 10% by 10 min and 21% by 30 min) with concomitant loss of $^3H$ label in the PI pool. The level of PIP was increased proportionately with increasing concentrations of cardiotoxin; for example, there was 10% increase with 10 µg/ml cardiotoxin and a 27% increase with 50 µg/ml cardiotoxin. The decline in the level of PI was also dependent on the concentrations of the toxin. There was, however, no significant change in the level of [$^3H$]$PIP_2$. In addition to the above effects there was a progressive loss of [$^3H$]inositol and [$^3H$]inositol phosphates into the medium along with loss of LDH from the cells. The time course of these events suggest that the primary effect of cardiotoxin was due to an enhanced rate of conversion of PI to PIP, rather than blockade of the conversion of PIP to $PIP_2$.

Stimulation of PI kinase activity in membrane preparations from MDCK cells. Phosphorylation by [$\gamma$-$^{32}P$]ATP of PI (to form PIP) was stimulated by addition of cardiotoxin to membrane preparations of MDCK cells. The stimulation was dependent on the concentration of the toxin. Measurement of specific membrane markers indicated that the enzyme activity was located predominantly in the plasma membrane.

Release of inositol phosphates in 2H3 cells: The release of inositol phosphates in cells stimulated with cardiotoxin (50 µg/ml) was small compared to that of antigen (10 µg/ml) stimulation: 4.2 $\pm$ 0.5 versus 26.4 $\pm$ 0.09%. In cells stimulated with cardiotoxin, there was a greater than 2-fold increase in the release of inositol bisphosphates and a 10-fold increase in inositol trisphosphates compared with untreated cultures. Interestingly there was a negligible increase in the inositol monophosphate. This is in contrast to the response of the cells to antigen stimulation in which the accumulation of inositol monophosphates predominates.

Time course of the release of [$^3H$]inositol phosphates, LDH and histamine from 2H3 cells in response to cardiotoxin: After 5 min following addition of 50 µg/ml cardiotoxin to 2H3 cell cultures there was a steady rate of release of [$^3H$]inositol phosphates over the period of 5-90 min. The toxin also caused a time-dependent release of histamine and LDH. Although the rate of release of LDH appeared to be faster than that of histamine, there was a general correlation between release of the two products.

Over a concentration range of 1-50 µg/ml, cardiotoxin caused a linear increase in the release of the [$^3H$]inositol phosphates from 2H3 cells. For release of LDH and histamine, maximal release appeared to have been reached at 30 µg/ml

The release of histamine but not LDH was totally dependent on external calcium. Thus, release of histamine appears to be through a $Ca^{2+}$ dependent process of exocytosis.

Significance to the Biochemical Research and the Program of the Institute: Our findings of the enhanced hydrolysis of membrane inositol phospholipids and the selective augmentation in the level of the PIP pool in cells stimulated indicate that the toxin has a unique stimulatory effect on phosphatidylinositol kinase. If further studies indicate that cardiotoxin has no effect on other enzyme activities that catalyze the interconversion of the inositol phosphates, the toxin would be a useful experimental probe in studies of inositol phospholipid metabolism. At the present time, no probes are available that act selectively at specific points of the phosphoinositide cycle.

Proposed Course of Project: As the molecular mechanism of the cytotoxic action of cardiotoxin is not clearly understood, more detail studies on the $Ca^{2+}$ ion dependency and the temporal relationship between secretion of histamine and leakage of LDH from cells stimulated with the toxin will be performed. Further, the mechanism(s) by which cardiotoxin selectively augmented cellular phosphatidylinositol-4-monophosphate pool will be investigated.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00993-01 LCP

PERIOD COVERED

October 1, 1986 through September 30, 1987

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

Phosphorylation of myosin heavy and light chains in stimulated basophils

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


P.I.: Russell Ludowyke Vist. Assoc. LCP NHLBI

Other:
Michael A. Beaven Chief, Section LCP NHLBI


COOPERATING UNITS (*if any*)

Drs. Robert S. Adelstein and Itzhak Peleg, LMC, NHLBI

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20892

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☐ (a1) Minors

☐ (a2) Interviews

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


A rat basophil leukemic cell line (RBL-2H3) exhibited phosphorylation of both the 200,000 and 20,000-dalton chains of myosin following antigenic stimulation. Cells were primed with specific IgE and labeled with 32P-orthophosphate and then stimulated with DNP-bovine albumin. Cells were then disrupted and myosin was selectively removed by immunoprecipitation with anti-platelet myosin antibodies. Scanning of the heavy chains in Coomassie-blue stained polyacrylamide gels revealed 1.1 µg of myosin per $10^6$ cells. Cells, stimulated at 30°C for 0.5 to 5 min, released increasing amounts of histamine, 32% of the total cellular histamine being released in 5 min. Scanning of autoradiograms suggested that the amount of radioactive phosphate associated with the myosin heavy and light chains increased 2-3 fold over that present in nonstimulated controls, during the same time period. Two- dimensional maps of tryptic digests indicated that the light chains were phosphorylated by myosin light chain kinase. and protein kinase C. Whether this phosphorylation of myosin within intact basophils plays a role in the secretory process, is under investigation.





PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description:

Objectives: Recent work in this and other laboratories has established that receptor mediated PI breakdown involves a complex array of biochemical changes. The pathways of the production and breakdown of the major pututative second messengers, inositol 1,4,5-trisphosphate ($IP_3$) and inositol 1,3,4,5-tetrakisphosphate ($IP_4$) has been clarified in the rat basophil leukemic (2H3) cell. The relationship between the production of these inositol phosphates and subsequent increase of intracellular $Ca^{2+}$ from internal and external stores has been described in other project reports from this Laboratory.

The breakdown of inositol phospholipids also initiates another stimulatory signal, the production of diacylglycerol (DAG) and stimulation of protein kinase (PKC). The role of this signal if any in the secretion of inflammatory mediatiors from 2H3 cells has not been investigated. In another secretory cell model, human platelets, PKC was shown to phosphorylate specific sites of the purified myosin light chain and thereby modify the ATPase and contractile performance of the myosin following phosphorylation by its most well known activator, myosin light chain kinase.

A role for the contractile proteins in the secretory process of mast cells and basophils has been suggested because a change in shape of the cells accompanies extrusion of intracellular granules. However, no evidence linking these processes is available. As antibodies to myosin were available studies were undertaken in collaboration with the Laboratory of Molecular Cardiology, NHLBI, to determine if any effect of PKC was apparent on the contractile proteins of the basophil 2H3 cells following antigenic stimulation. Analogous studies with platelets have shown that changes in phosphorylation of myosin light chain is accompanied by changes in shape of the cell. This project complements Project No. Z01 HL 0409-01 MC.

Methods Employed: Cell culture procedures and priming of the cells with specific antibody for subsequent release (antigen induced) have been described in previous reports. The myosin, which constitutes 0.1% of the total basophil cellular protein, is separated from the cell extract by precipitation with an anti-human platelet antibody. The effect of various stimulatory and inhibitory procedures and the time course of phosphorylation was then followed after separation of the 200,000 dalton heavy chain x 20,000 dalton light chain on SDS-polyacrylamide gels. To find out which sites on the heavy and light chain are phosphorylated and therefore which kinase is responsible the areas corresponding to the light and heavy chain of myosin were out from the gels, and the labeled peptides were separated by a 2-dimensional electrophoretic and chromatograph technique. To determine the content or stoichiometry of phosphorylation, the peptides were separated first by isoelectric focusing and then by SDS polyacrylamide gel electrophoresis to prepare 2-dimensional peptide maps. The position of the radioactive material was determined by autoradiography. Release of histamine was determined as described in Project Report No. Z01 HL 00937-04 LCP.

Major findings: i) Autoradiograms revealed that in unstimulated cultured basophilic cells both the 200,000 dalton heavy chain and then 20,000 dalton light chain are partially phosphorylated. Following antigenic stimulation, there is an additional incorporation of [$^{32}$P]phosphate into both chains. The additional incorporation

is evident in one minute and reaches a maximum between 10 to 20 minutes. Thereafter the extent of labeling is decreased.

ii) Two dimensional peptide maps, of the light chains showed that in the basal unstimulated state, phosphorylation is evident at only one site, which is the target site of myosin light chain kinase. After antigen stimulation, there are transient changes in the state of phosphorylation at this site but between 1 and 2.5 min, another [$^{32}$P]phosphorylated site appears which is a serine (1 or 2) site whose phosphorylation is catalyzed by PKC. This site becomes the predominant site of [$^{32}$P] phosphorylation and reaches maximal levels of phosphorylation by by 10 to 20 min.

iii) When the [$^{32}$P]phosphorylated peptides were cut from the gels, it was apparent that the changes in radioactivity in the PKC site correlated with the release of histamine. However, with a concentration of antigen that was optimum for secretion the release process was largely complete before the levels of [$^{32}$P]phosphorylation showed a marked decline.

iv) The stoichiometry of light chain phosphorylation reveals that 40% of the myosin is phosphorylated in unstimulated cells, whereas almost 100% is phosphorylated in stimulated cells.

v) Earlier studies had revealed that prolonged exposure (24 hr) of 2H3 cells to phorbol ester (PMA) which is known to activate protein kinase C, leads to loss of PKC (see Project Report No. Z01 HL 00973-03 LCP). After 24 hr incubation with 50 nM PMA, the time course of histamine secretion is delayed and there is a much reduced phosphorylation at the myosin light chain site catalyzed by PKC.

Significance to Biomedical Research and to the Program of the Institute: We have shown that activation of a protein kinase C - dependent phosphorylation of light chain - myosin occurs in antigen stimulated 2H3 cells. This phosphorylation shows a temporal relationship to secretion but a causal relationship has yet to be established. The activation of protein kinase C is a consequence, presumably, of the hydrolysis of inositol phospholipids which is a well defined event in the 2H3 cells.

Proposed Course of Project: As outlined in accompanying project reports, we now have a detailed understanding of the early stimulatory events in 2H3 cells and of how these events lead to the generation of a $Ca^{2+}$ signal and activation of protein kinase C. This project is to examine the distal stimulatory events that are related to activation of protein kinase C and of $Ca^{2+}$-calmodulin-dependent myosin kinase. Future work will investigate the stoichiometry of phosphorylation at the sites phosphorylated by the action of protein kinase C and myosin kinase. The relationship between these phosphorylation events and secretion will be critically examined by use of a variant of the RBL-2H3 cell that lacks protein kinase C (see Project Report No. Z01 HL 00975-03 LCP) and by our ability to deplete protein kinase C by prolonged exposure of the 2H3 cell to PMA. The possible involve-

ment of myosin in antigen-stimulated exocytosis of granule from 2H3 cells will be examined in a preliminary way by looking for the presence of myosin binding proteins in the perigranular membranes.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 00994-01 LCP

PERIOD COVERED

October 1, 1986 through September 30, 1987

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.: Y. Singh | Guest Researcher | LCP | NHLBI |
| Others: | | | |
| 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. 20892

| 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.)

Cardiac myocytes isolated from 2 day old rats were cultured in Primaria flasks for 8 days.They were incubated with various analogs of daunomycin and adriamycin at clinically relevant micromolar concentrations for up to 96 hr. The cell death was monitored using a number of parameters such as loss of cell ATP, depletion of cell GSH, leakage of adenine nucleotides and cytoplasmic enzyme such as lactic acid dehydrogenase. In general the cells lost ATP and GSH before the leakage of enzyme and adenine nucleotides. Modification of adriamycin and daunomycin molecule markedly increased or decreased the toxicity depending on the modification. The most toxic compound was found to be cyano morpholino analog of adriamycin. These analogs were also tested for their antitumor activities against mouse leukemia L1210 cells. These drugs also depleted cell ATP and GSH before cell death. However, their effect on the growth of the L1210 cells were somewhat independent of their effects on ATP and GSH. In general an increase in antitumor effects induced by certain chemical modifications also increased cardiotoxicity of anthracycline analogs. However certain modification in the structure increased the cardiotoxicity without an increase in their antitumor effect and vice versa. It can be concluded from these studies that the mechanism of cell death in cardiac myocytes and tumor cell may involve the effect on ATP synthesis while the effect of anthracycline on cell growth may involve other factors such as intercalation with DNA and inhibition of DNA synthesis.

Project Description:

Objectives: The main aim of this study is to investigate the mechanism by which anthracycline analogs induced cardiotoxicity. Another objective is compare the structural requirements of anthracyclines to induce cardiotoxicity and to suppress tumor cell growth at clinically relevant submicromolar concentrations.

Methods Employed: The method for isolation of cardiac myocytes from 2 day old rats and their maintenance in tissue culture has been described in detail in earlier reports. Mouse leukemia cells L1210 were grown as cell suspension and the effects of various anthracycline analogs were tested on cell growth as well as cell death. Trypan blue staining was used as a marker of cell death and cell growth as estimated up to 96 hr by enumeration of viable cells which excluded trypan blue.

Major Findings: In general cardiac myocytes lost cell ATP and GSH before the leakage of cytoplasmic enzyme such as LDH and leakage of adenine nucleotides. By using clinically relevent submicromolar concentrations the cell death occurred after 48-72 hr whereas cell ATP and GSH were lost as early as 24 hr. Thus the sequence of events obtained with these concentrations were the same as that obtained with much higher concentrations of anthracyclines. Certain structural modifications of the anthracycline molecular markedly increased the toxicity of the parent compound. The most toxic analog of the 20 analogs so far studied appears to be the cyanomonopholine analog of adriamycin. Both adriamycin and daunomycin appear to be equally toxic when tested at submicomolar concentrations. Modification of the 4'-hydroxyl group in the sugar moiety of anthracycline markedly potentiate the cardiotoxicity replacement of 4-hydroxyl group in the anthracycline ring by a hydrogen also resulted in increased toxicity. When these analogs were tested for their antitumor effects in L1210 cells, most of the analogs tested decreased cell ATP and GSH before causing cell death. However, these analogs inhibited cell growth at concentrations much lower than those required for depletion of cell ATP and GSH. Thus the mechanism of inhibition of the growth of tumor cells does not appear to be due to a decrease in either ATP or GSH amd may be different from that causing cell death. With a several analogs modifications that increased the antitumor effect there also resulted in a corresponding increase in the cardiotoxicity. However, some modifications in the structure increased cardiotoxicity without any increase in antitumor effect and vice versa.

Significance to Biomedical Research and to the Interest of the Institute. These findings indicate that even though the mechanism of cell death induced by anthracycline in cardiac myocytes and tumor cells may be the same, the mechanism on cell growth may be different. It may thus be possible to modify the structure of adriamycin and daunomycin in such a way to produce analogs which have high therapeutic ratios. The development of a simple *in vitro* models for comparing cardiotoxicity and the antitumor effects of anthracycline analogs may aid in selecting analogs for tests in animals. This will greatly reduce the number of animals used for their testing.

Proposed Course of Project. We propose to modify the procedures used to assess toxicity of the drugs in cells in long term culture in order to minimize the *in vivo* time course of events. In general cardiotoxicity *in vivo* develops over a period of 13-15 weeks with doses of 1 mg/kg/week. It is now possible to use these doses and test the effect on cardiac myocytes over 6-10 week period. We have been able to maintain these cultures over 4 week periods without any change in their beating rates. It is of interest to investigate the effect of anthracycline analogs over an extended period for 4-8 weeks. We also plan to test other analogs with this system. We have been able to demonstrate that loss of ATP from cardiac cells which occurs prior to cell death is the main mechanism of anthracycline induced cell death. We propose to examine this aspect in the long term culture to see if loss of ATP preceeds cell death. We also propose to determine whether the effects of anthracycline are accumulative and thereby result in a delay in the inhibition of ATP synthesis in cardiac myocytes. This could explain why cardiotoxicity is delayed *in vivo*.

Publications:

Shirhatti, V., George, M., Chenery, r. and Krishna, G.: Structural requirements for inducing cardiotoxicity by anthracycline antibiotics: Studies with neonatal rat cardiac myocytes in culture. Toxicology and Applied Pharmacology 84: 173-191, 1986.

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Maheshwari, R.K., Husain, M.M., Singh, V.K., Krishna, G. and Friedman, R.M.: Mechanism of interferon inhibition of enveloped viruses. Asian Congress of Pharmacology, in press.

ANNUAL REPORT OF THE

LABORATORY OF CHEMISTRY
SECTIONS ON CHEMICAL STRUCTURE AND STRUCTURAL
NUCLEAR MAGNETIC RESONANCE
NATIONAL HEART, LUNG AND BLOOD INSTITUTE

October 1, 1986 through September 30, 1987

Synthesis is a renewed interest in the laboratory and will now be the major activity of Dr. Tappey Jones, formerly of the College of William and Mary, with whom we have been collaborating on an informal basis over the last 8 years. His activities include, but are not limited to stereospecific synthesis of nitrogen heterocycles and monoterpenoid substances. He replaces Dr. S. Miller who now works with Dr. R. Brady (NINCDS) on a permanent basis.

Nuclear Magnetic Resonance continues to be the major activity of the laboratory, employing 4-6 persons under Ferretti and Highet. All spectrometers (360,300, 200 MHz) are in satisfactory operating condition and are continually being upgraded to the state of the art. A Microvax 2 computer has been provided for rapid data processing and molecular mechanics. It will be interfaced to the Cray supercomputer (NCI) in Frederick.

Ferretti's group is involved in research on AIDS related drugs, specifically peptide T and related analogs. This work was funded in part by an intramural targetted antiviral AIDS program. The aim is to establish relationships between molecular structure and antiviral activity via NMR.

NMR studies on actinomycin D analogues show that they exist in the C conformation while only A conformation analogues have biological activity: DNA intercalation may be involved (A. Mauger, Washington Hospital Center). Similar conformation studies on the tachykinins (substance P, physalaemin, uperolein and neurokinin A) have shown them to yield conformational information only in methanol and this may yield information on receptor binding. (R. Jernigan and D. Covell, LMB:NCI)

The conformations of the second loop of transforming growth factor (TGF) and epidermal growth factor (EGF) have been compared by NMR. These two loops are implicated in receptor binding. Only the second loop fragment of EGF binds to the receptor and triggers biological activity. These NMR studies have demonstrated significant differences in the conformation of the second loops of TGF and EGF which may be responsible. (B.R. Brooks, DCRT).

An oligopeptide and its anti-sense peptide from the 1-13 fragment of ACTH were synthesized and their individual resonances in water assigned. Upon mixing, the expected interactions were minimal but the experiment will be repeated in trifluoroethanol to enhance interaction affects. (E. Najim, Howard Hughes MRI).

Two AIDS related peptides (C. Pert NIMH) have been studied in detail. One inhibits cell infectivity the other does not. The only difference in NMR

is the amide chemical shift perhaps resulting from orientation of the tyrosine side chain.

Several studies on theory and precision of NMR measurements have been conducted (G. Weiss, DCRT and R. Byrd, FDA) since this aspect is critical in evaluating results from NMR measurements. Using NMR 2-D-nuclear Overhauser techniques, a series of methylene-linked phenethylamides has been studied to gain insight about hindered rotation around the amide linkage. Multiple resonances were observed whose exact explanation remains to be unknown.

Solid state studies on a Vitamin E dimer have shown that its solution fluxion, hypothesized by us 10 years ago, but only verified this year through spin labeling studies, does not occur in the solid state. HPLC analyses on an optically active column at 0°C actually resulted in resolution of the two optically active forms and their rotations and subsequent racemization were subsequently measured. Several similar resolutions of trace quantities of optically active insect pheromones were conducted using either chiral columns or derivatization with optically active and UV-absorbing reagents. The absolute configuration of one complete class of fire ant alkaloids (the solenopsins) was finally established in this manner.

NMR studies using COSY and NOESY methods have established the structure and sterochemistry of a truxillic acid derivative (H. Ziffer & A. Bax, NIDDK).

Several insect constituents have been elucidated using NMR and/or GC-MS. These include a dimethyldecadienol from butterfly (J. Wheeler, Howard U.), a homofarnesene from an ant and the 3-pentyl ester of 2-methyl-3-hydroxybutyric acid from the granary weevil. The latter insect is responsible for millions of dollars of damage annually. We have recently synthesized this pheromone as well as several analogues (W. Burkholder, USDA).

Synthesis of the toxic metabolites of bromobenzene is in progress ( S. Lau U. of Houston and T. Monks, Georgetown U.)

In x-ray crystallography, Dr. Silverton has determined the crystal structures of the important AIDS drugs, DDC, DDA, and AZT. The aim here is to provide real energy minima for use in molecular mechanics investigations. He also completed, or is nearing completion, structures of a taxol rearrangement product, triglycine, epoxysteroid, and a tetrahydrodibenzanthracene diol with collaborators from this area. Beyond x-ray, Dr. Silverton has equipped his IBM PC-AT to carry out extensive CINDO (quantum mechanics) and MM2 (molecular mechanics) calculations, producing results equivalent to those obtained previously only on a DEC VAX 11-750.

In mass spectrometry, we have acquired a Finnegan ion trap which all allow us to study parent/daughter/grandaughter relationships among ions. In addition, it is the most sensitive mass spectrometer available today and should allow us to pursue extremes in trace analysis of low molecular weight compounds. The Cf-252 spectrometer continues to produce excellent results and is now being increasingly used to confirm the structure of synthetic peptides. L. Pannell (NIDDK) maintains an active interest and is a valuable collaborator in this effort.

As one example of the success of the Cf-252 system, crisamycin C was shown to have 16 AMU more than crisamycin A after failing to yield results with all other mass spectrometric systems (W. Russell, Wakseman Institute, N.J.). Furthermore, an extensive series of alkylated cyclodextrins have been analyzed successfully. These substances are required by FDA to be carefully characterized since they are used to increase drug solubility (J. Pitha, NIA, Baltimore).

Demethyldiazepam, reported to occur naturally last year (DeBlas, Stoneybrook) has been confirmed as occurring in hog brain and moldy potatoes by the Hoffman-LaRoche laboratories. We are now carrying out experiments designed to suggest modes of its biosynthesis.

In a mass spectrometric study of the enzymatic reactions of succinic semi-aldehyde, 2-hydroxyglutaric acid was unexpectedly detected. This led to the elucidation of the important new redox system; succinic semialdehyde $\rightleftharpoons$ 4-hydroxybutyrate/2-ketoglutarate $\rightleftharpoons$ 2-hydroxyglutarate. Perturbations of this system may be expected to manifest themselves as inborn errors of metabolism (E. Kauffman, NIDDK).

Three unusual amino acids, isolated from Streptococcus lactis, were characterized and subsequently synthesized: (25,7S)-N(5)-(1-carboxyethyl)-ornithine, (25,8S)-N-(6)-(1-carboxyethyl-lysine and N(5)-acetyl-ornithine. The first two had not previously been found in nature. The stereochemical configuration of the carboxyethyl derivatives were determined by C-13 NMR studies, and the biosynthetic route to these compounds was identified by a combination of in vivo labeling and mass spectroscopy.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01002-13 CH

PERIOD COVERED

October 1, 1986 - September 30, 1987

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

Application of Nuclear Magnetic Resonance

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

Edward A. Sokoloski    Chemist    NHLBI CH

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NHLBI NIH, Bethesda, Maryland 20892

| 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.)

Nuclear Magnetic Resonance is being used to study the hindered rotation of some novel N-Methylphenethylamide dimers. The results from line shape analyses and the new two-dimensional nuclear Overhauser technique will be compared.



PHS 6040 (Rev. 1/84)

GPO 913-918

Project Description

We are examining the Nuclear Magnetic resonance spectral characterictics of a series of molecules originally synthesized by members of this laboratory. These compounds were made as models to study doubly-charged ions in mass spectroscopy. The materials are dimers of N-methylphenethylamide with various length methylene chains joining the two molecules at the carbonyl function.

The molecules exhibit very slow hindered rotation about the amide bond which makes them excellent candidates for study by the newer two dimensional nuclear Overhauser spectroscopy. We hope to obtain the thermodynamic parameters from the 2D method and compare the results obtained with results from the older line shape method.

$C_6H_5CH_2CH_2N(CH_3)COCH_3$ A 1

$C_6H_5CH_2CH_2N(CH_3)CO(CH_2)_nCON(CH_3)CH_2CH_2C_6H_5$ A 2 ⟶ A 10

Observations

All materials show multiple resonance lines for the N-$CH_3$ moeity. The number and separation of these lines varies depending on the number of bridging methylenes present (see chart).

PROTON

| Sample | (ppm) | D( ) Hz. |
|---|---|---|
| A 1 (M=0) | 2.85,2.93 | 14.2 |
| A 2 (M=0) | 2.61-2-65,2.0-2.93 | 65.5 |
| A 3 (M=1) | 2.78-2.88,2.93-2.94 | 20.1 |
| A 4 (N=3) | 2.86-2.88,2.93-2.94 | 10.2 |
| A 7 (N=5) | 2.82.86,2.93 | 14.3 |
| A 10 (M=2) | 2.89-2.92,2.93 | 6.5 |

Values separated by dashes are resonances that coalesced first during preliminary variable temperature experiments, usually between 100 and 120°C. The peaks with larger chemical shift divergence had coalescence temperatures greater than 170°C. In order to obtain the exact coalescence temperature for line shape measurements, experiments with sealed tubes and a solvent other than $CDCl_3$ will be needed.

Several other facts emerged from the data:

1) There is a decrease in the number and seperation of these resonance when increasing the number of bridging methylenes. If the multiplicity of lines from one resonance were due to hindered rotation about the amide bound alone, there should be very little difference in all the molecules studied. This portion of the molecule remains unchanged through the series.

2) Intensities of the lines appear to be equal. This indicates that the rotamer conformations are equally populated and of nearly equal stability. Other resonances, particularly the N-$CH_2$, show the same multiple resonances as the methyl group but do not show the same intensities. This should not be the case.

In addition to the proton data, we have run carbon-13 spectra on all these materials. They too, show multiple lines for each carbon atom. Due to the lack of compling interactions, they are somewhat simpler in appearance. Hetero-correlation spectroscopy will help to understand the proton spectra. No variable temperature work, as yet, has been done in carbon spectroscopy. The separation of the carbon resonances of the N-$CH_3$ and N-$CH_2$ are given below.

| | -N-$CH_3$ (Hz) | -N-$CH_2$- (Hz) |
|---|---|---|
| A1 (n=o) | 176 | 143 |
| A2 (n=o) | 164 (?) | 211 |
| A3 (n=1) | 149 | 107 |
| A4 (n=3) | 125.6 | 84 |
| A7 (n=5) | 129 | 85 |

PUBLICATIONS

1. Yang, Y.M., Sokoloski, E.A., Fales, H.M. and Pannell, L.K. Californium-252 Plasma Desorption Mass Spectrometry of Skin Lipids Positive and Negative Ions Formed by Attachment Processes. Biomed. and Environ. Mass Spect. 13, 489-92, 1986.

2. Shirhatti, V., Sokoloski, E.A., Eng. S., Hench, S., Riccardi, F. and Krishna, G. A Simple Method for the Assay of Bordetella Pertussis Adenylate Cyclase Employing $^{31}P$ Nuclear Magnetic Resonance Spectroscopy. J. of Cyclic Nucleotide and Protein Phosphorylation Research. 11 (2): 137-47, 1986.

3. Jacobson, K.A., Pannell, L.K., Kirk, K.L., Fales, H.M. and Sokoloski, E.A. Californium-252 Plasma Desorption Mass Spectrometry as an Aid in the Synthesis of a Series of Adenosine and Xanthine Conjugates. J. Chem. Soc. Perkin Trans. I. (2) 43-49, 1986.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-HL-01003-15-CH

PERIOD COVERED

October 1, 1986 to September 30, 1987

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 | S.P. Miller | Staff Fellow, NHLBI:CH |
| | P. Cohen | Chemist, NHLBI:CH |

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: |
|---|---|---|
| 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 variety of natural products have been studied using GC-MS, NMR and HPLC, including the granary weevil pheromone, 3 new amino acids from S. lactis, a vitamin E-dimer, and numerous insect constituents. Experiments on the possible biosynthesis of valium have been intiated and several studies using the Cf-252 spectrometer have been successful.

1. As reported last year, demethyldiazepam (demethylvalium) was found in mammalian brain, even in human tissues placed in storage before the drug was developed (DeBlas et al, Stonybrook). The identification using mass spectrometry was unequivocal, and this suprising finding has now been corroborated by the Hoffman-LaRoche labs who also find other related compounds in moldy potatoes. A series of experiments designed to determine possible biosynthetic pathways for such an unusual chlorine-containing base has been undertaken using hippuryl anthranilamide as a model. No cyclizations under biological conditions have been observed to date.

2. Three unusual amino acids, isolated from Streptococcus lactis (carboxyethylornithine, carboxyethyllysine and N-acetylornithine), not previously known in nature, were characterized and subsequently synthesized. The biosynthetic route to these compounds was identified by a combination of in vivo labeling and mass spectrometry.

3. The structure of the Granary weevil, a serious economic pest was elucidated as the 3-pentyl ester of 2-methyl-3-hydroxybutyric acid of the R*, S* configuration using a combination of mass spectrometry and NMR. The substance has been synthesized via a Reformatsky reaction and the expected isomer is active. Several of its closely related pentyl esters have now also been synthesized to determine the structural specificity of the organism's receptor sites.

3. (a) The Cf-252 spectrometer system continues to solve many intractable problems. Crisamycin C was thought to be related to crisamycin A but other MS methods failed to explain the difference. Cf-252 clearly showed an increase of 16 amu from oxygen in both positive and negative ion modes, corroborating NMR results and allowing a structure to be deduced (W. Russell, Waksman Inst.).

(b) A series of peptides of ever-increasing molecular weight is being successfully run on the Cf system. Early problems with surface layer adhesion are being studied in detail and 15KV accelerating volts is routine.

(c) The LeBeyec digitizer, allowing multiple stops, has arrived and is being interfaced by DCRT. The reflectron has still not be delivered.

4. The vitamin E dimer is an in vivo oxidation product of Vitamin E. Over 10 years ago we suggested that it was a novel fluxional (A A) system Magnetization transfer experiments have now confirmed this and using chiral HPLC columns at 0°C we have resolved it into its enantimers (H. Lloyd, CH) and measured its rotation. It is not clear that its racemization involves fluxion and, since a free radical can be detected at high sample concentrations, other mechanisms are possible, and are being studied. We are also attempting to prepare a more readily handled naphthyl derivative to study this interesting phenomenon.

PUBLICATIONS

1. Liav, A., Goren, M.B., Yang, Y.M., Fales, H.M. Synthesis of 4,6-anhydro-6' 0-mycoloyl- and 4,6-anhydro-6'0-corynomycoloyl-(D-galactopyranosyl-D-galactopyranoside). *J. of Carbohydrate Research,* 155 223-28, 1986.

2. Yang, Y.M., Sokoloski, E.A., Fales, H.M. and Pannell, L.K. Californium-252 Plasma Desorption Mass Spectrometry of Skin Lipids. Positive and Negative Ions formed by Attachment Processes. Biomed. and Environ. Mass Spec. 13, 489-92, 1986.

3. Jones, T.H., Blum, M.S. Andersen, A.N. and Fales, H.M. 2-Ethyl-5-Alkypyrrolidines in the Venom of an Australian (Monomorium Species) Ant. J. Chem. Ecol. in press.

4. Jacobson, K.A., Pannell, L.K., Kirk, K.L., Fales, H.M. and Sokoloski, E.A. Californium-252 Plasma Desorption Mass Spectrometry as an Aid in the Synthesis of a Series of Adenosine and Xanthine Conjugates. J. Chem. Soc. Perkin Trans I. 2143-49, 1986.

5. Sangameswarian, L., Fales, H.M., Friedrich, P,. and DeBlas, A.L. Purification of a benzodiazepine from bovine brain and detection of benzodiazepine-like immunoreactivity in human brain. Neurobiology. 83, 1-5, 1986.

6. Yang, Y.M., Lloyd, H.A., Pannell, L.K., Fales, H.M., Macfarlane, R.D., McNeal, C.J. and Ito, Y. Separation of the Components of Commercial Digitonin Using High Performance Liquid Chromatography and Centrifugal Countercurrent Chromatography. Biomed. and Environ. Mass Spec. 13, 439-45, 1986.

7. Blum, M.S., Morel, L. and Fales, H.M. Chemistry of the Mandibular Gland Secretion of the Ant Componotus vagus. Comp. Biochem. Physiol. 86, No. 2, 251-52, 1987.

8. Fales, H.M. Recent Developments in Mass Spectrometry. Beltsville Symposia Research Instrumentation for the 21st Century. Agriculture Research. Vol. 11. Martinez-Nichov, N.Y.

9. Young, A.M., Blum, M.S., Fales, H.M. and Bian, Z. Natural History and Ecological Chemistry of the Neotropical Butterfly Papilio anchisiades (Papilionidae). J. of the Lepidopterists Society 40 (1), 36-53, 1986.

10. Tomalski, M.D., Blum, M.S., Jones, T.H., Fales, H.M., Howard, D.F., Passera, L. Chemistry and Functions of Exocrine Secretions of the Ants Tapinoma melanocephalum and T. erraticum. J. Chem. Ecol. 13, No. 2, 253-63, 1987.

11. Dhariwal, K.R., Yang, Y., Fales, H.M. and Goren, M.B. Detection of Trehalose Monomycolate in Mycobaeterium leprae Grown in Armadillo Tissues. J. of General Microbiology. 133, 201-09, 1987.

12. Rinderer, T.E., Blum, M.S., Fales, H.M., Jones, T.H., Buco, S.M., Lancaster, V.A., Danka, R.G. and Howard, D.F. Nest Plumdering, Allomones of the Fire Bee Trigona (Oxytrigona) mellicolor [1,2]. J. Chemical Ecol. In press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01004-16 CH

PERIOD COVERED
October 1, 1986 to September 30, 1987

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: H. A. Lloyd 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: 1 | PROFESSIONAL: 1 | 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 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).



PHS 6040 (Rev. 1/84)

GPO 913-918

Chiral Separations by High Pressure Liquid Chromatography

1) Vitamin E Dimer Analog

A reexamination of the structure of the synthetic Vitamin E dimer analog (where the isoprenoid side chain of the natural tocopherol has been replaced by a methyl group) led to the separation of the two enantiomers of the dimer by HPLC. This was accomplished using a Pirkle A-type column (ionically bound N-(3,5-dinitrobenzoyl D-phenylglycine) and a solvent consisting of 2.5% propanol-2 in hexane and cooling both column and solvent to 0°C. The pure enantiomers were collected (at 0°C) and their optical rotation measured at that temperature (by H.M. Fales, CH:NHLBI). The rate of racemization at various temperatures was studied.

2) HPLC sparation of d,l-solenopsin enantiomers:

The absolute configuration of dihydro-trans-2-methyl-6-tridecylpiperidine (solenopsin B), the hydrogenation product of an alkaloid isolated from a fire ant, Solenopsis invicta, (by T.H. Jones, College of William & Mary) was determined with the aid of HPLC. The retention times of derivatives of the natural product, the synthetic trans d,l and synthetic trans-R,R enantiomer (synthetic materials obtained from D. Taber, U. of Delaware) were compared. It was concluded that the natural alkaloid has the trans R,R configuration. The alkaloids were derivatized with tetraacetyl-D-glucopyranosyl isothiocyanate and their solution injected into a $C_{18}$ Radial Pak cartridge flushed with acetonitrite water.

3) The enantiomeric separation of pyrrolidine alkaloids of other Solenopsis ants was accomplished both with chiral derivatization on a $C_{18}$ column or without derivatization on a chiral Pirkle column.

Synthetic Drugs: These projects have been continued from last year.

1) Mass spectrometric studies on synthetic dopaminergic and hypotensive drugs (with S.L. Evans, Meharry Medical College).

2) Animal growth promoters (with A.H. Khan, FDA). The use of certain chemicals (i.e. nitrofuranes, nitroimidazoles) as antiparasitic agents or rapid growth promoters in poultry and cattle often results in the presence of undesirable residues in animal tissues. A study of these residues (of the drugs and their metabolites) by high pressure liquid chromatography and mass spectrometry is in progress.

C. Insect Pheromones

Collaboration was continued with M.S. Blum, University of Georgia; R.R. Snelling, Natural History Museum of Los Angeles; J.L. Clement, University of Paris; A. Hafetz, Tel-Aviv University. The chemical compositions of glands of a large number of ants, bees and other insects sent by these researchers, have been determined by GC-MS. In particular most of the ants in the genus Myrmecocysts native to the West and South West USA have been looked at. Out of more than 60 volatile compounds identified only one had never been found previously in other insects: 2-hexyl-2-decenal.

Other Pheromones: Two new collaborative projects have been initiated.

1) Volatile pheromones from snakes (with P.J. Weldon, Texas A & M University). Examination of the secretions from the scent gland of rattlesnakes and yellow-bellied sea snakes has been undertaken.

2) Study of the volatile components in urine and scent gland fluids relative to ovulation in peccaries (Tayasu tajacu) with J.M. Packard, Texas A & M University

PUBLICATIONS

1. Yang, Y.M., Lloyd, H.A., Pannell, L.K., Fales, H.M., MacFarlane, R.D., McNeal, C.J. and Ito, Y. Separation of the Components of Commercial Digitonin Using High Performance Liquid Chromatography and Centrifugal Counter Current Chromatography. Identification of the Products by Californium-252 Desorption Mass Spectrometry. Biochem. Mass. Spectrom. 13, 439-445 (1986)

2. Blum, M.S., Snelling, R.R., Duffield, R.M., Hermann, H.R. and Lloyd, H.A. Mandibular Gland Chemistry of Componotus (Myrmothrix) Abdomanilis: Chemistry and Chemosytematic Implications in Advances in Myrmecology. Edited by J.C. Trager E.J. Brill Publishing Co. Leiden and N.Y. 1987 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01005-16 CH

PERIOD COVERED

October 1, 1986 to September 30, 1987

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: 1.1 | PROFESSIONAL: 1.1 | OTHER: 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.)

Solid state and computational work of the Laboratory of Chemistry, NHLBI concerns structural and configurational studies of biologically-interesting compounds. Drug action, chemical synthesis and biological function of natural and synthetic drugs, peptides and nucleotides have been investigated.



PHS 6040 (Rev. 1/84)

GPO 914-918

Project Description

a) Computer studies. The IBM PC-AT is now equipped with a Definicon DS132 coprocessor. Benchmark programs, both local and published, indicate that the processing speed is equal to that of a Digital Equipment Corporation VAX 11-750, as had been hoped.

Two major chemical programs have been successfully implemented on the coprocessor: CINDO (quantum mechanics) and MM2 (molecular mechanics). A non-trivial amount of reprogramming was necessary especially to MM2 but the programs now work as expected and at satisfactory speeds.

Ancilliary programs have been written to allow the above programs to communicate with main-frames and other more conventional programs on the PC

It seems very likely that PCs will replace ordinary terminals in this laboratory for communication with main frame computers both on the campus and for time-sharing services. Experience this year indicates that a PC is just as likely to be in use for this purpose as is a stand-alone terminal. Graphics resolution still leaves something to be desired but it seems almost certain that satisfactory resolution will be available within a few months.

Work, initiated last year, on an actinomycin-related peptide lactone has been extended using standard mini-computer programs. Analysis of the results to determine differences between physical measurement and calculation is in progress. Some agreement is highly satisfactory but significant differences may exist.

The newly implemented MM2 program has been used on a calibration run on cyclo-octene and results are being studied. To this end programs have been written to extract any desired calculation result for display on main-frame graphics. Extension to larger ring systems is being planned.

b) Drugs of value and interest in AIDS therapy (with Dr. F. Quinn, NCI). With a view to providing at least local energy minima for use in molecular mechanics investigation of the action of deoxynucleotides, the crystal structures of dideoxycytidine (DDC) and dideoxyadenosine (DDA) have been determined. A paper on the work has been submitted. It had been intended to also study the drug azidothymidine (AZT) but, when our work was at a preliminary stage, it was discovered that a crystal structure was almost complete in another laboratory. Results of the AZT structure will be obtained and we make our results available to the other investigators. Several interesting compounds in this class are being prepared and will be investigated if suitable crystals can be obtained. However, competition for crystals of such drugs can be fierce.

c) Synthetic nucleotides (with Prof. W. Stec, Polish National Academy of Sciences). This compound is the largest single molecule ever attacked by direct methods in this laboratory. After considerable work, it was decided that the X-ray data could be improved. New, purer crystals were obtained and the large data set (some 8000 reflections) was remeasured. As yet a successful solution has not been obtained but work is still in progress.

d) Taxol Rearrangement Product (with Prof. David Kingston, Virginia Polytechnic and State University, Blacksburg). Initial difficulties and problematic analyses indicating chlorine have been traced to the tendency to form weak solvates with most solvents. The initial solvent was methylene chloride which was readily partially lost. It has been found that an ethyl acetate crystal is less labile and should be stable in a closed capillary.

e) Triglycine (with Dr. Y. Hiyama, NIDDK). The crystal structure of this compound was studied some years ago (with Dr. A. Bavoso), University of Naples (Italy) and the results were used in conjunction with solid state NMR in an attempt to explain the multiplicity of crystal forms. While the second major habit gave crystals too small for a full crystallographic investigation, it was possible to establish that the unit cell was quite different from the previous form and the combined results have clarified the situation. It is interesting that the different crystal structures have a probable cause similar to that we deduced for p-fluorophenylalanine in that a bilayer with hydrophobic surfaces is formed.

f) Epoxysteroid (with Prof. C.H. Robinson, Johns Hopkins University and Dr. Victor Ekhato, this laboratory). The study was carried out to prove the structure and configuration. No difficulties had been expected but the structure proved quite intractible and only yielded to rather refined techniques. The configuration was not as expected.

g) Carcinogens (with Dr. Hari Yagi and Dr. Donald Jerina, NIDDK). The absolute configuration of 1,2,3,4-tetrahydrodibenzanthracene-3,4-diol has been established by solving the crystal structure of its 1-menthyl diester. The compound is of a type where alleged absolute configurations have been reported based on the non-robust Hamilton test. In the present case, it is quite apparent that naive approaches have about a 50% chance of being incorrect and the result will be discussed in a forthcoming paper.

h) Other activities. Referee for several journals, reviewer for granting agencies (including recently Research Council/Medical of Canada), invited lecturer, Washington Crystal Colloquium, Johns Hopkins University. NIH user member-at-large of the Source Selection Board for procurement of the new central computer facilities (chairman: Dr. William Raub, Deputy Director, NIH).

PUBLICATIONS:

1. Dumont, R., Brossi, A. and Silverton, J.V. Facile Conversion of Natural Colchicine into (+)-Congeners and (+)-Enantiomers Including 2-Demethyl Analogues. J. Organic Chemistry. 2515-21, 1986.

2. West, D.X., Scovill, J.P., Silverton, J.V. and Bavoso, A. Nickel (II) Complexes of a Thiosemicarbazone Prepared from 2-Acetylpyridine. Transition Met. Chem. 11, 123-31, 1986.

3. Burke, T.R, Jr., Jacobson, A.E., Arthur. E., Rice, K.C., Silverton, J.V., Simonds, W.F., Streaty, R.A. and Klee, W.A. Probes for narcotic receptor mediated phenomena. cis-(+)-3-Methylfentanyl isothiocyanate. J. Med. Chem. 29 6: 1087-93, 1986.

4. Ito, S., Masaji, K., Ziffer, H. and Silverton, J.V. Preparation of Chiral allylic alcohols using Rhizopus nigricans. Use of the Harada-Nakanishi Exciton chirality method for verifying configuration and assignment of allylic alcohols. Can. J. Chem. 65, 574-82, 1987.

5. Misra, R., Pandey, R.C., Hilton, B.D., Roller, P. and Silverton, J.V. Structure of Fredericamycin A: Spectroscopic and Chemical Characterization J. of Antiobiotics 40, in press.

6. Silverton, J.V., Brossi, A. and Dumont, R. Structure and absolute configuration by several methods of 2-acetyl-2-demethoxythiocolchicine. Acta Cryst. C 43, in press.

7. Awaya, H., May, E.L., Aceto, M.D., Harris, L.S. and Silverton, J.V. Hexahydro-1H-1-pyrindenes from Acid Rearrangement of 9-alkyl-5-(m-methoxy phenyl)-2-methyl morphans. J. Med. Chem. 52, 947-50, 1987.

8. Mauger, A.B., Ferretti, J.A., Gallagher, K.S. and Silverton, J.V. Two-dimensional NMR and computer modeling studies of conformers of an actinomycin-related peptide lactone. Proceedings 10th American Peptide Symposium, May 1987, Ed. Garland A. Marshall (in press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01006-16 CH

PERIOD COVERED
October 1, 1986 - September 30, 1987

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 |
| OTHER: | I.V. Ekhato, Ph.D. | Visiting Fellow | |

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 |
|---|---|---|
| 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.)


NMR studies have demonstrated the structures of a truxillic acid, and of terpenoids from a bee, Nomada sp., and from an ant, Manica rubida. Synthetic studies have been initiated to prepare the nephrotoxic metabolites of bromobenzene.





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

Structural Studies by Two-Dimensional NMR Techniques.

Studies in collaboration with Drs. Herman Ziffer and Ad Bax of NIDDK have established the structure and sterochemistry of a truxillic acid derivative formed by the dimerization of methyl p-nitrocinnamate. Since the molecule lacks symmetry, only two of the eleven possible isomers need be considered. COSY and NOESY spectra show it to be the isomer with one aromatic ring trans to the other substituents on the cyclobutyl system.

Insect Pheremones.

A collaborative study with Professor James Wheeler of Howard University has established the structure of a metabolite of a bee, Nomada sp. Proton and COSY spectra show it to be 3,7-dimethyldeca-2,6-dien-1,10-diol, a material previously identified in the pheremonal secretion of the Queen butterfly, Donus gilippus berenice.

Terpenoids from the gasters of the ant Manica rubida have been studied in collaboration with Dr. Tappey Jones of the College of William and Mary. The material of molecular weight 204 is farnesene, while that of molecular weight 218 is a homofarnesene, an ethyl group evidently appearing in the place of one terminal methyl.

Nephrotoxic Metabolites of Bromobenzene

In a collaborative study with Dr. Serine Lau of the University of Houston and Dr. Terry Monks of Georgetown University, liver metabolites of bromobenzene have been shown to include the three isomeric glutathionyl bromohydroquinones, the structures of which are established by their proton spectra. A diglutathionyl derivative is also formed, but the structure of this last, much the most toxic, is not explicitly known. 5,6-Diglutathionylbromohydroquinone appears to be excluded by the carbon-13 spectra. In the absence of promising spectral methods to distinguish between the other isomers, a synthetic program has been initiated. These studies are currently in an exploratory phase, directed towards the preparation of the various ethylthiobromohydroquinones as model materials. As studies on the corresponding free mercaptans have shown them to be nephrotoxic, these target models will also be suitable subjects for toxicity studies.

Award: American Society for Pharmacology and Experimental Therapeutics 1985 Best Paper Award.

PUBLICATIONS:

1. Daly, J.W., Whittaker, N., Spande, T.F., Highet, R.J., Feigl, D., Nishimori, N., Tokuyama, T., and Myers, C.W. Alkaloids from Denrobatid Frogs: Structures of two $\omega$-Hydroxy Congeners of 3-Butyl-5-Propylindolizidine and Occurrence of 2,5-Disubstituted Pyrrolidines, J. Nat. Products Chemistry, 49, 265-80, (1986).

2. Jones, T.H., Highet, R.J., Don, A.W. and Blum, M.S., The Alkaloids of Chelaner antarcticus. J. Org. Chem., 51, 2712-16, (1986).

3. Stack, M.E., Mazzola, E.P., Page, S.W., Pohland, A.E., Highet, R.J., Tempesta, M.S., and Corley, D.G., Mutagenic Perylenequinone Metabolites of Alternaria Alternata, J. Nat. Prod., 49, 866-71, (1986).

4. Highet, R.J. The Formation of an Unusual Addition Compound from 3,5-Dipyrrolidinophenol. J. Org. Chem., 51, 3231-32, (1986).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01027-05 CH

PERIOD COVERED

October 1, 1986 to September 30, 1987

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, Ph.D. | Chemist | NHLBI CH |
| Donald Davis, Ph.D. | Senior Staff Fellow | NHLBI CH |
| Kathleen S. Gallagher, MA. | IPA Fellow | NHLBI CH |
| Susan Sumner, Ph.D. | Staff Fellow | NHLBI CH |
| Kyou Hoon Han, Ph.D. | Visiting Fellow | NHLBI CH |


COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.5 | .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.)


Research involves the development and application of multiple pulse Fourier transform methods in nuclear magnetic resonance spectroscopy, including solvent suppression and two dimensional techniques. Applications include conformational properties of peptides and small proteins in solution, studies in the precision of the methodology, and physical properties of peptide-protein complexes




PHS 6040 (Rev. 1/84)

GPO 914-918

1) Conformational Studies of Intermediate Molecular Weight Peptides.

Studies on newly synthesized peptide lactones related to actinomycin D have been initiated. These new peptides are demethylated analogs of previously studied peptide lactones (i.e., glycine in place of sarcosine and valine in place of N-methylvaline). The demethylated analogs of actinomycin D are biologically inactive. Preliminary studies indicate that these analogs exist only in the C conformation in contrast to the methylated derivatives which can exist in the A or C conformation. Only the A conformation shows any biological activity in the actinomycin D molecules. The relationship between the dimerization of these analogs in solution, their ability to intercalate with DNA, and their biological activity is under investigation. These studies are being carried out in collaboration with A.B. Mauger, Washington Hospital Center and J.V. Silverton, CH/NHLBI.

Conformational studies on the series of tachykinins have been carried out under various conditions of solvent and temperature. The tachykinins are linear peptides which contain 10-13 amino acid residues whose carboxyl termini are amidated and end with the sequence Phe-X-Gly-Leu-Met $NH_2$. Here X is the only residue of the carboxyl end which may vary. The individual tachykinins have many common features of their biological activity and it would be interesting to correlate structure and activity. The peptides that have been studied include substance P, kassinin, physalaemin, uperolein, and neurokinin A. The general feature shown by the entire series is that they have definable conformations in methanol whereas they appear quite flexible in water solution. Two-dimensional rotating frame cross relaxation spectroscopy (ROESY) was used to obtain distance constraints between individually assigned hydrogen atoms of each of the peptides in methanol. Substance P has been studied in the greatest detail since the overall features of the entire series appear to be similar. Supplementary geometric constraints were derived from the vicinal spin-spin couplings. These constraints in combination with an energy minimization program were used to compute an all-atom structure for substance P. The similarity in biological activities coupled with structural similarities found for the entire series in methanol suggests that this or a similar conformation might be required for receptor binding. These studies are being carried out in collaboration with Kathleen S. Gallagher (CH:NHLBI), Susan C.J. Sumner (CH:NHLBI) Amrie Otto (CH:NHLBI) Robert L. Jernigan (LMB:NCI), and David Covell (LMB:NCI).

Two cyclic fragments of human transforming growth factor (TGF-$\alpha$); a cyclic TGF (21-32) and a cyclic $Ala^{21}$ - TGF (16-32) have been studied. Also a cyclic fragment of epidermal growth factor (EGF) 16-33 has been synthesized. Since a recent study on pattern recognition in the genetic code has suggested that one of the two possible receptor binding sites in EGF involves the residues from 24 to 29, the conformational behavior of these peptides has been studied in water solution. These studies are being carried out in collaboration with Kyou Han (CH:NHLBI) and Bernard R. Brooks (DCRT). Geometric constraints were obtained from the two dimensional laboratory frame (NOESY) and rotating frame (ROESY) experiments and structures were obtained using these results in combination with dynamic simulation and energy minimization procedures.

A recently proposed theory of molecular recognition has been derived from the observation that in the genetic code, codons for hydrophilic and hydrophobic

amino acids on one strand of DNA are generally complemented by codons for hydrophobic and hydrophilic amino acids on the other strand, respectively. The average tendency of codons for hydrophilic amino acids is complemented by codons for hydrophobic amino acids. Thus, one might expect that an oligopeptide and its complement (usually the anti-sense one since it is read on the 5' to 3' direction) might bind to each other. It has been proposed that such interactions might represent a model for peptide-receptor binding. As an initial test the anti-sense complementary peptide for the 1-13 fragment of adrenocorticotropic hormone was synthesized. The spectra of the sense and anti-sense peptides were run separately and their resonances completely assigned in water solution. Neither peptide showed any evidence of structure. The rotating frame cross relaxation spectrum of the mixture in water solution showed some differences over the spectra of the individual components. These data are currently being analyzed in detail similar experiments are also being carried out in trifluoroethanol. These studies were performed in collaboration with Elias Najem (Howard Hughes Medical Research Institute)

Two AIDS related octapeptides have been obtained from the laboratory of Candace Pert and have been analyzed in detail. These two peptides are d-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-$ThrNH_2$ and d-Ala-Ser-Thr-Thr-Thr-Asn-Tyr-d-$ThrNH_2$. In Vitro assays performed in the laboratory of Dr. Pert indicate that the first peptide inhibits cell infectivity whereas the second peptide does not. Also both peptides are predicted by minimum energy calculations to have a turn between the fourth and fifth Thr residues. The ROESY spectra as well as the temperature dependent peptide N-H chemical shifts in methanol on both peptides are consistent with this prediction. The only spectroscopically observable difference between the two peptides is the chemical shift difference between the amide and groups in the active and inactive species. This result may suggest a difference in the orientation of the tyrosine side chain with respect to the threonine amide. It is difficult to understand, however, how this difference could account for the observed differences in cell infectivity These studies are being carried out in collaboration with Kathleen S. Gallagher, Susan C.J. Sumner (CH:NHLBI), and David Covell (LMB:NCI).

2) Theory and Precision in Nuclear Magnetic Resonance Spectroscopy.

Studies are continuing in the determinations of errors of the measurements of NMR parameters in two-dimensional Fourier spectroscopy. These studies are being carried out in collaboration with G.H. Weiss (PSL:DCRT) and R.A. Byrd (FDA). A review on the measurement of spin lattice relaxation times has been completed and will appear shortly. Procedures for estimating the errors in diagonal and cross peak volumes and strategies for minimizing these errors have been developed. The object of this investigation is to determine the precision in the estimate of internuclear distances used in peptide and protein structure determinations. The effects of various apodization functions on the precision of the cross peak volumes has been investigated in detail.

3) Development of New Methods for Spectral Assignment and Structure Determination In NMR Spectroscopy

The use of cross polarization methods for the study of carbon-13 spectra of crystalline solids has been implemented on the Varian XL-300. As an application

of the method, a dimer of Vitamin E was investigated (H. Fales and H. Lloyd CH: NHLBI). The complete assignment of the solid state carbon-13 spectrum was made. In solution the molecule shows fluxional properties whereas in the solid state there is no evidence for similar behavior. The solid state spectra, however, do suggest the existence of two slightly different conformational forms in the crystal state. The nature of these conformational differences is under investigation. Preliminary investigations have begun on new pulse methods for improving the quality of spectra obtained from a homonuclear Hartman-Hahn cross polarization experiment. Such experiments should reduce the intensity of the diagonal peaks and provide better suppression of solvent resonances. These new techniques involve the use of filtering of pulses at the end of the mixing period. Theoretical analyses of the methods are simultaneously being carried out.

PUBLICATIONS

1. Weiss, G.H., Ferretti, J.A. and Boyd, R.A. Accuracy and Precision in the Estimation of Peak Areas and NOE Factors II. The Effect of Apodization. J. Mag. Resonance 71, 97 (1987).

2. Weiss, G.H. and Ferretti, J.A. Optimal Design of Relaxation Time Experiments. J. Magn. Resonance 71, 97 (1987).

3. Han, K-H., Niu, C-H., Brooks, B.R., Ferretti, J.A. and Roller, P.P. Structures of Human TGF - Fragments Using Two-Dimensional Proton Magnetic Resonance Spectroscopy and Computer Simulation. Proc. 10th Annual Peptide Symposium (in press).

4. Mauger, A.B.,Ferretti, J.A., Gallagher, K.S. and Silverton, J.V. Two-Dimensional NMR and Computer Modeling Studies of Two Conformers of an Actinomycin Related Peptide Lactone. Proc. 10th Annual Peptide Symposium (in press).

Annual Report of the Clinical Hematology Branch
National Heart, Lung and Blood Institute
October 1, 1986 to September 30, 1987

The research of this Branch is directed toward understanding the underlying causes and developing effective treatment for major hematological disorders, primarily those affecting the red cell. 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. There are several major areas of research in the Branch. The molecular mechanisms of globin gene regulation during development are being defined and new strategies to manipulate HbF synthesis are under investigation. Retroviral vectors are being designed and tested to introduce expressing globin genes into bone marrow cells in vivo. Another major area of investigation is the study of growth factors and expression of their genes during normal and abnormal hematopoiesis. The use of anti-sense nucleic acid sequences to modulate gene expression is being explored as a tool for study of gene function and as a potential therapeutic intervention. The pathogenises and treatment of aplastic anemia and the role of viruses in bone marrow failure are other major areas of investigation.

Patients with either severe beta-thalassemia or sickle cell anemia could benefit from increased production of fetal hemoglobin. Fetal hemoglobin ($HbF=\alpha_2\gamma_2$) produced in utero, is rapidly replaced during the perinatal period with the adult type of hemoglobin ($HbA=\alpha_2 \beta_2$). At the gene level, this switch reflects turn off of the gamma globin gene and turn on of the beta globin gene. If both beta genes are defective, the switch leads to the onset of disease.

Regulation of the switch involves the interaction of trans-acting factors (proteins) with control (cis-active) DNA sequences linked to the globin genes. Our research has focused on identification of such cis-acting sequences (Individual Projects "Regulation of hemoglobin switching during devlopment: characterization of globin gene promoters" and "Identification of cis and trans-acting elements that regulate human gamma gene expression"). An upstream activating sequence has been defined in the gamma globin gene promoter and an enhancer has been identified downstream from the two gamma globin genes. The promoter sequence is sufficiently defined to allow initiation of efforts to identify and purify trans-acting factor(s) that binds and activate this region. Further evidence that this region is critical is the demonstration that point mutations increase promoter function and lead to elevated synthesis of HbF in adults. Globin genes have been introduced into fertilized eggs of mice. The transgenic animals that were obtained express the human gamma and beta genes with developmental and tissue specificity. This model will be developed to fully explore the function of regulatory sequences from the gamma globin gene region during development.

The dihydrofolate reductase gene is constitutively expressed in all cells. It is modulated by regulatory mechanisms different from those of the globin genes. This promoter is part of a methylation

free island in chromatin although we have learned that only a small segment of this island must be undermethylated for gene expression (Individual Project:"Characterization of the gene for human dihydrofolate reductase"). Expression of this gene is modulated during the cell-cycle. Recent evidence supports a hypothesis that modulation is regulated at the level of mRNA elongation.

During the past year we have reinitiated efforts designed to manipulate HbF production in vivo (Individual Project:"Pharmacological manipulation of HbF synthesis"). A phase I-II trial of hydroxyurea in patients is designed to determine the response frequency and appropriate dose to maximize the therapeutic effect. The use of physiological regulators of hematopoiesis such as erythropoietin and interleukin 3 to perturb erythroid progenitor and precursor kinetics provides a potential alternative avenue for more physiological induction of HbF synthesis. These substances are currently being tested in Rhesus monkeys.

An alternative approach to genetic therapy is the introduction and expression of genes in bone marrow cells. We have made significant progress during the past year in that a vector has been designed that routinely results in gene expression in primary hematopoietic cells in vivo (Individual Project:"Use of viral regulatory sequences to facilitate gene expression and analysis of gene function"). Red cells containing human beta globin chains were detected in 100% of animals for 6-8 weeks following repopulation. The target cell for gene insertion in these experiments appears to be a multi-potential hematopoietic progenitor with limited self-renewal capacity. Future experiments will focus on 1) enhancing the level of expression by addition of cis-active control sequences to the viral vector and 2) increasing the probability that gene transfer will be effected in stem cells with more significant self-renewal potential.

Modification of the surface of retroviral particles to target to specific cells may enhance the potential of such vectors for genetic therapy (Individual Project:"Modification of retroviral targeting by hybrid envelope proteins"). Disappointing results were obtained with the initial constructions in which the IL3 coding sequences were inserted into the envelope protein reading frame. New proteins have been designed and the constructions initiated in an effort to overcome this problem. We have expanded these efforts to include the HIV envelope protein as it is now known from the work of others that this protein interacts with a specific receptor on T-lymphocytes. Development of a packaging cell line with the capacity to target recombinant vectors to lymphocytes seems likely to be feasible.

Molecular characterization of hematopoietic growth factors and their receptors has opened many new avenues of investigation into the mechanisms of hematopoiesis. The c-fms proto-oncogene encodes the receptor for monocyte-colony stimulating factor (M-CSF), a primary regulator of monocyte and macrophage formation and function. We have shown that modifications at the C-terminal end of the c-fms protein may convert it to a transforming gene (Individual Project:Function of proto-oncogenes in human hematopoietic cells). In the future, we plan

to study the structure of the c-fms gene in primary human hematopoietic malignancies to learn whether analogous mutations occur in these cells.

The 5q⁻ syndrome is a refractory anemia characterized by the presence of an abnormal chromosome 5 in bone marrow cells. An interstitial deletion of the long arm of 5 has removed the genes for several hematopoietic growth factors and receptors. During the past year we have assigned the newly discovered IL3 gene to this region and shown that it is within 9kb of the gene for GM-CSF. These hematopoietic regulators have very analogous functions on hematopoietic cells and are apparently part of a multigene family. The mechanism whereby the 5q⁻ deletion leads to refractory anemia is uncertain. We are testing the hypothesis that this deletion uncovers "silent" mutations on the opposite cytogenetically normal chromosome 5. A systematic effort has been initiated to test the function of the individual growth factor and growth factor receptor genes in the cells of these patients.

The strategy of retroviral mediated gene transfer provides another avenue to explore the mechanisms of action of hematopoietic growth factors in normal hematopoiesis and of their mutated forms in hematopoietic malignancies. We have created retroviral vectors that transfer and express the coding sequences for mouse IL3 and GM-CSF. Endogenous expression of these sequences allow cells to replicate and differentiate independent of exogenous growth factors. Data has been obtained indicating that the growth factor is produced and secreted into the endoplasmic reticulum where it binds to the receptor, leading to an action that mimics the affects of exogenous ligand.

Introduction of growth factor encoding sequences into hematopoietic stem and multi-potential progenitor cells leads to the emergence, in mice, of a myeloproliferative syndrome that resembles chronic myelogenous leukemia (Individual Project:"The effect of V-abl and IL3 genes on hematopoietic stem cell differentiation"). Marked elevation of the white blood cell count and organ enlargement occurs because of excessive production of morphologically normal cells. This experimental model has thereby provided direct evidence that a "single mutational hit" in a gene involved in growth regulation can cause a myeloproliferative disorder. We are testing the hypothesis that a second hit in a different gene can lead to the emergence of acute leukemia. Once this model is established one can test complementing mutations in several genes in an effort to understand the experimental basis of hematopoietic neoplasia.

Nucleic acids sequences complementary to messenger RNA (anti-sense sequences) may inhibit gene expression by blocking RNA processing or translation. We have shown that c-fos and c-myc anti-sense RNA inhibits cell proliferation and interferes with differentiation of teratocarcinoma cells. In an alternative approach, we have used synthetic DNA oligomers to inhibit c-myc gene expression in human promyelocytic cells in culture, leading to inhibition of proliferation and enhanced differentiation (Individual Project:"Use of anti-sense RNA or DNA to inhibit gene expression"). Our results with

anti-sense inhibition of gene expression have encouraged us to initiate a major effort to utilize anti-sense RNA to inhibit replication of the HIV virus (Individual Project:"Inhibition of HIV replication in T-lymphocytes by anti-sense RNA sequences"). The strategy that we have developed involves combining anti-sense technology with construction of retroviral vectors that we hope ultimately may be targeted to T-lymphocytes. This laboratory is in a unique position to undertake this effort as we have expertise in all of these methodologies.

A major focus of clinical interest of the laboratory is aplastic anemia. Previous studies have implicated activated T-lymphocytes as being an important pathogenetic mechanism in such patients. Gamma interferon is thought to be the mediator of a suppressive effect on hematopoiesis. During the last year we have made a preliminary observation suggesting that interleukin I production is deficient in monocytes of these patients. This observation assumes major significance in that interleukin I is now known to be identical to hematopoietic growth factor, H1, that acts on the very earliest hematopoietic progenitors and perhaps on stem cells. Of interest is the fact that gamma interferon inhibits production of growth factors by monocytes. Experiments are planned to test this regulatory loop in great detail in aplastic anemia patients.

The Branch has conducted several clinical trials of therapy in these patients. A multi-center cooperative trial is now complete and the data has been analyzed (Individual Project:Lymphocytes and lymphokines in aplastic anemia). Approximately 50% of patients with severe aplastic anemia had significant hematological improvement; the duration of therapy was not a major variable in determining response rate. Of equal interest is the demonstration that 5 of 15 patients with severe aplastic anemia, refractory to ATG treatment, had a significant hematological response to the combination of cyclosporine and prednisone. These data suggest additional clinical studies of combined modality therapy. Hematopoietic growth factors have been molecularly cloned and are now available in amounts to allow clinical trials. We have shown that GM-CSF accelerates return of granulocyte numbers to adequate levels following bone marrow transplantation in a Rhesus monkey model. A protocol has been designed to test the activity of GM-CSF in previously untreated patients with severe aplastic anemia.

The B19 parvovirus, discovered only 10 years ago, has been shown to be the cause of transient aplastic crisis of chronic hemolytic disease and fifth disease, a common childhood exanthem. Work in this laboratory has shown that it can also occasionally cause pancytopenia in immunodeficient individuals. This observation has initiated an effort to determine whether B19 parvovirus, or related viruses, could be implicated in the pancytopenia frequently observed in patients with AIDS. A complete transcriptional map with the B19 parvovirus has been obtained and the coding sequences for the major proteins identified (Individual Project: B19 (human) parvovirus). Transfection of the B19 genome into non-erythroid cells suggest that the pattern of transcription may differ between erythroid and non-erythroid cells.

Transcriptional pausing at the center part of the genome in non-erythroid cells precludes synthesis of the proteins required for encapsidation and virus production. Future work will focus on clarification of this novel genetic mechanism. A new project has also been initiated to investigate the role of Epstein-Barr virus in bone marrow suppression (Individual Project:Epstein-Barr virus and aplastic anemia). This project was based on the clinical observation of bone marrow aplasia after virus infection in three individuals.

A major collaborative study has been initiated to define the chromosomal location of the locus, mutant alleles of which can lead to hypertrophic cardiomyopathy (Individual Project:"Mapping of the hypertrophic cardiomyopathy locus"). The availability of unique probes that detect highly polymorphic markers, through collaborative interactions, make this a feasible undertaking. The study involves a collaborative effort on the part of the Cardiology and Hematology Branches in a NHLBI and the genetic expertise provided by the Clinical Epidemiology Branch of the NCI.

In summary, the work of the Branch has expanded greatly and refocused on several problems of immense importance. Genetic therapy for hemoglobin disorders has become a realistic goal and is being vigorously pursued. Our knowledge of anti-sense inhibition of gene expression has suggested that this might be useful in treatment of AIDS and we have greatly expanded our effort to determine whether this is true. Hematopoiesis has become ammenable to the tools of molecular biology as the genes for hematopoietic regulators are defined and characterized. Many new avenues of investigation have been opened and our energies have been redirected to take advantage of these opportunities to understand human disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 02208 13 CHB

PERIOD COVERED
October 1, 1986-September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Iron Chelation and Transfusional Hemachromatosis

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

PI: Arthur W. Nienhuis, Chief, Clinical Hematology, CHB, NHLBI
Others: Patricia Griffith, Clinical Nurse Specialist, CHB, NHLBI
W.F. Anderson, M.D., Branch Chief, LMH, NHLBI
Gary Brittenham, M.D., Division of Hematology, Cleve. Gen Hosp.
H. Strawczynski, M.D., Dir., Chronic Care Clinic, Montreal Children's Hospital, Montreal, Quebec, Canada
Evan Tucker, M.D., Senior Investigator, CB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Hematology

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: 0.5 | PROFESSIONAL: 0.5 | OTHER: 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. Desferoxamine is administered by subcutaneous infusion and iron removal is determined by measurement of the serum ferritin and periodic non-invasive measurement of liver-iron concentration. Clinical status is evaluated by standard parameters including non-invasive testing of cardiac and endocrine function as indicated by the patients age and risk category. The study is designed to document the natural history of severe beta thalassemia, treated effectively with regular transfusions and chelation therapy tailored to the patient's clinical status.



PHS 6040 (Rev. 1/84) GPO 914-910

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. Our studies have documented the utility of various parameters for determining the efficacy of chronic chelation therapy. Most important is a periodic non-invasive measurement of liver-iron concentration as a guide to the effectiveness of iron removal. Endocrine testing, particularly estimation of glucose tolerance and function of the pituitary-gonadal axis, provides important information with regard to the actual efficacy of chelation therapy. Our experience over the last eight years has resulted in the design of therapeutic regimens specifically tailored to each individual's risk category.

Methods:

Patients who participate in these studies include those with transfusion dependent congenital or acquired anemia who require regular blood transfusions to sustain life. In addition, the study includes selected patients with idiopathic hemochromatosis who exhibit significant cardiac dysfunction.

Clinical evaluation of organ function includes the following.

1. Heart: An estimate of cardiac size by chest x-ray and electrocardiographic analysis. Echocardiography is used to determine anatomical dimensions and left ventricular function at rest. In selected patients, radionuclide angiography is performed to determine the functional reserve of the left ventricle during excercise.

2. Endocrine evaluation includes specific testing of the pituitary, thyroid, adrenal, pancreatic islets, and gonad function by baseline measurements and various provacative 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. 37:1671, 1983).

Results:

Repeat liver iron concentrations have been determined in approximately 2/3 of the 80 patients who are part of this study. A clear correlation has emerged between the compliance with chelation therapy (number of vials used per year), and the change in liver iron concentration between measurements. Noncompliant patients exhibit progressive iron loading whereas compliant patients exhibit variable degrees of iron unloading depending on the intensity of treatment. In addition, a correlation has been noted between clinical status and liver iron concentration although occasional patients who are compliant with therapy exhibit clinical evidence of iron overload, eg

diabetes mellitus. Continued follow-up will be necessary to determine the clinical benefit of this therapy.

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 therapy 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. Long term follow up of patients in various risk categories will define those most appropriate for gene therapy protocols as these are devised.

Publications:

1. Nienhuis, A.W., and Wolfe, L.C.: The Thalassemias: Disorders of Hemoglobin Synthesis. In, Hematology in Infancy and Childhood. Nathan, D.G. and Oski, F.A., Eds. W.B. Saunders, Philadelphia. 3rd edition, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02307 08 CHB

PERIOD COVERED

October 1, 1986-September 30, 1987

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)

PI: David M. Bodine, Ph.D., Staff Fellow, CHB, NHLBI

Others: Arthur W. Nienhuis, Chief, Clinical Hematology Branch, NHLBI
Stefan Karlsson, Associate Fellow, CHB, NHLBI
Deborah Rothenhoefer, Biologist, CHB, NHLBI
Thalia Papayannopoulou, Univ. of Washington, Seattle, WA
George Stamatoyannopoulos, Div. Med. Genetics, Univ. of Washington, Seattle

COOPERATING UNITS (if any)

University of Washington, Seattle, Washington

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

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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.)

Retroviral gene transfer is a powerful tool for studying gene expression as well as a model for gene replacement therapy. Previous work by this laboratory has shown that derivatives of the N2 retroviral vector will efficiently transfer genes to murine erythroid cell lines and early hematopoetic progenitor cells. We have modified the N2 vector to contain a human $\beta$ globin gene. Recombinant viruses packaged by $\psi$2 packaging cells were used to infect mouse bone marrow cells. These cells were used to repopulate histocompatible genetically anemic $\underline{W/W^v}$ mice. Cells were injected directly after infection, or following an additional 48 hours of selection with G418, as the N2 $\beta$ globin virus also contains a neomycin resistance gene. At various times post infection the peripheral blood of these animals was sampled and analyzed for human $\beta$ globin gene expression by immunoflourescent staining.

Four weeks post infection the animals repopulated with unselected marrow were 80% repopulated with the infected marrow (as monitored by genetic markers) and 45.5% (5/11) were expressing human $\beta$ globin in up to 20% of the peripheral blood cells. The animals repopulated with G418 selected marrow were 30% repopulated with the infected marrow and 100% (12/12) were expressing human $\beta$ globin in up to 20% of the peripheral blood cells. We are continuing to monitor the expression of human $\beta$ globin in these mice.

Project Description:

Objectives:
The objectives of this project are to introduce various genes into the bone marrow stem cells of mice and to have these transduced genes expressed at appropriate levels in the differentaited descendents of these cells. Retrovirus mediated gene transfer is an efficient method to introduce new genetic material into the somatic cells of whole animals, and as such serves as a model for gene therapy in humans. Previous work in this laboratory has shown that derivatives of the N2 retrovirus will infect early murine hematopoetic progenitor cells. We have modified this virus to contain a human $\beta$ globin gene. This virus has been shown to infect and express the human $\beta$ globin gene appropriately in murine erythroleukemia cell lines. We have used this virus to infect mouse bone marrow cells and have analyzed the expression of the human $\beta$ globin gene for 12 weeks following transplantation.

Methods:

A human $\beta$ globin gene containing ~ 250 bp of 5' flanking sequence and ~400 bp of 3' flanking sequence was inserted into the Xho I site of pXM6, a plasmid containing the N2 retrovirus. This recombinant plasmid contains a neomycin resistance gene controled by the retroviral promoter elements and a human $\beta$ globin gene controled by it's own promoter elements. This plasmid was used to transfect the packaging cell line $\psi$-2. These cells contain a defective retroviral sequence that produces all of the proteins necessary for the packaging of retroviruses, but cannot itself be packaged. Thus retroviral transcripts from the transfected N2 $\beta$ globin genome are packaged into viral particles which are released into the supernatant and can be used to infect other mouse cells.

Subconfluent plates of $\psi$-2 cells producing the N2 $\beta$ globin virus were overlaid with 1 x $10^7$ bone marrow cells from C57BL/6J - +/+ mice. Following 24 hours of co-culture the cells were removed from the producer cells and 2 x $10^6$ cells were injected into the tail veins of WBB6F1 - $W/W^v$ mice. WBB6F1 - $W/W^v$ mice have a severe macrocytic anemia caused by a deficiency of bone marrow stem cells. These mice can be cured by transplants of normal histocompatible bone marrow without irradiation. Because C57BL/6J mice have a different hemoglobin marker, it is possible to moniter the repopulation of the host with the infected marrow.

Alternatively, following the infection step, the cells were preselected for neomycin resistance by culturing for 48 hours in 2 mg/ml G418 (actual concentration). Following selection, 2-5 x $10^6$ viable cells were injected into $W/W^v$ hosts.

At regular intervals after transplantation the peripheral blood was sampled and analyzed for expression of the human $\beta$ globin gene by immunoflourescent staining using a monoclonal antibody directed against human $\beta$ globin. The repopulation of the $W/W^v$ host by the

infected C57BL/6J marrow was monitered simultaneously by measuring the amount of donor hemoglobin in the peripheral blood.

Results:

Two weeks post transplantation the DNA content of CFU-S colonies revealed that about 30% of the CFU-S derived from unselected cells and 100% of the CFU-S derived from selected cells contained a retrovirus. Four weeks post infection the animals transplanted with unselected marrow were 80% repopulated with the infected marrow and 45.5% (5/11) were expressing human β globin in up to 20% of the peripheral blood cells. The animals repopulated with G418 selected marrow cells were 30% repopulated with the infected marrow and 100% (12/12) were expressing human β globin in up to 20% of the peripheral blood cells. At 12 weeks post infection the animals transplanted with unselected marrow were 100% repopulated with the infected marrow and 18.2% (2/11) were expressing human β globin in up to 10% of the peripheral blood cells. The animals repopulated with G418 selected marrow cells were 30% repopulated with the infected marrow and 41.7% (5/12) were expressing human β globin in up to 10% of the peripheral blood cells. We are currently monitoring the expression of human β globin in two additional sets of mice.

Proposed course of project:

Currently we are involved in projects designed to increase the expression of the transduced β globin gene. We are constructing a series of N2 β globin vectors that include a positive regulatory sequence located 3' to the human β globin gene. The positive regulatory sequence has been inserted into the N2 β globin vector in the LTR (replacing the viral enhancer region), 5' to the β globin gene, and in it's natural location 3' to the gene.

In addition we are attempting to increase the repopulating capacity of the selected marrow cells by using larger numbers of viable cells and alternative methods of tissue culture designed to maximize the retention of pluripotent hematopoetic stem cells.

Publications:

1. Karlsson, S., Van Doren, K., Schweigher, S.G., Nienhuis, A.W. and Gluzman, Y. (1986) Stable Gene Transfer and Tissue-specific Expression of a Human Globin Gene Using Adenoviral Vectors, EMBO J 5: 2377-2385.

2. Karlsson, S., Papayannopoulou, T., Schweiger, S.G, Stamatoyannopoulos, G., and Nienhuis, A.W. (1986) Retroviral-mediated Transfer of Genomic Globin Genes leads to Regulated Production of RNA and Protein, Proc. Natl. Acad. Sci. USA 84: 2411-2415.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 02310 07 CHB

PERIOD COVERED

October 1, 1986-September 30, 1987

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

Characterization of the Gene for Human Dihydrofolate Reductase

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


PI: Takashi Shimada, M.D., Visiting Associate

Others: Arthur W. Nienhuis, M.D., Branch Chief


COOPERATING UNITS *(if any)*

LAB/BRANCH

Clinical Hematology

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20892

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☐ (a1) Minors

☐ (a2) Interviews

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


The promoter of the constitutively expressed human dihydrofolate reductase (DHFR) gene has been characterized by deletional analyses. Various deletional mutants of the promoter sequence were linked to the bacterial chloramphenicol acetyltransferase (CAT) gene and introduced into Hela cells. The relative activity of the CAT constructs was determined by a newly developed quantitative assay system. Two separate positive regulatory elements, from-612 to -360 and from-111 to -72, relative to the major cap site, were identified in the 5' flanking region. A nuclear runoff assay revealed that the transcriptional rate of exon 1 is about 10 fold higher than that of exon 2, indicating that the transcriptional elongation is blocked within intron 1 sequence. This evidence and the characteristic chromatin structure of this region previously defined suggest the possiblity that the expression of the DHFR gene is regulated at the level of mRNA elongation during the cell cycle.

Project Description:

Objectives:

Dihydrofolate reductase (DHFR), a key enzyme of one carbon metabolism, is essential for cell growth and cellular metabolism. The DHFR gene is among the house keeping genes, which are constitutively expressed at a low level in all cells. The level of expression, however, is modulated during the cell cycle and increases on growth stimulation. It appears that the mode of regulation of the DHFR gene is distinct from that of tissue specific specialized genes. Another interesting point is that the DHFR gene is often amplified in cells selected for resistance to methotrexate (MTX). The purpose of this study is to define the structure and mechanism of regulation of the DHFR gene and to investigate the mechanism of the gene amplification.

We have previously cloned and characterized the functional human DHFR gene and three intronless DHFR pseudogenes. The functional gene is about 30 kilobase long and consists of six exons and five introns. One major transcriptional start site and three polyadenylation sites have been identified. The sequence of the 5' promoter region is highly homologous to the corresponding region of the mouse DHFR gene; it is extremely rich in guanosine and cytosine residues. Only this promoter region was found to be undermethylated, while the remaining 30 kilobase gene was completely methylated. Detailed study of the chromatin structure of the promoter region using various nucleases showed that the normal nucleosome array is interrupted at the 5' end of the DHFR gene by about 900 base pairs of nucleosome-free DNA, to which several nuclear proteins bind in a DNA sequence specific manner.

The DHFR minigene composed of all the coding sequences and 1.2 kilobase of the 5' flanking sequence transformed DHFR- CHO cells at a significant frequency. The integrated minigene was usually amplified in CHO cells up to 500 copies in the absence of methotrexate. The chromatin structure in the promoter region of the minigene was very similar to that of the endogenous human DHFR gene. Methylation of the minigene in vitro reduced transforming frequency to 10-20% of control. In cells transformed with the methylated minigene only a region corresponding to the minimal promoter was specifically demethylated, while other parts of the vector, including the DHFR coding and plasmid sequence, remained completely methylated.

The RNA mapping study and the in vitro transcription assay showed the presence of RNA molecules transcribed from the opposite DNA strand for the DHFR transcript, indicating that the promoter of the DHFR gene functions in a biodirectional manner. Another weak promoter was identified in the upstream region. Transfection assay using various deletion mutants of the DHFR minigene showed the 72 bp 5' flanking sequence is sufficient for DHFR gene expression.

We have made a further characterization of the DHFR gene promoter using a highly sensitive and quantitative CAT assay system. We have also started a series of experiments to elucidate the molecular basis of the cell cycle regulation of the DHFR gene expression.

Methods:

Plasmid Construction:

A series of deletion mutants of the DHFR promoter was linked to the bacterial chloramphenicol acetyltransferase (CAT) gene by the standard DNA recombinant technique.

Quantitative Transient Assay:

Various CAT constructs and the RSV gpt plasmid were co-introduced into Hela cells using the standard $CaPO_4$ coprecipitation method. After two days, the CAT and the gpt activities were assayed by a newly developed rapid and sensitive method. The CAT activities were normalized for equal transfection efficiency using the gpt activity as an internal standard.

Nuclear Runoff Assay:

Isolated nuclei from methotrexate resistant Hela cells were incubated with 32P labeled ribonucleotide triphosphate in vitro. RNA was extracted from the reaction mixture and hybridized to various cloned probes.

Major Findings:

Because the promoter activity of the constitutively expressed genes such as DHFR gene, is considerably less than that of viral or tissue specific genes, a highly sensitive and quantitative assay system is required for functional analyses of the promoter. The chloramphenicol acetyltransferase (CAT) assay system has been widely used for this purpose. However, variability in transfection efficiency from one dish to another and in different experiments is a major problem for accurate comparison. To overcome this problem, we cointroduced  second marker gene, Rans sarcoma virus promoter driven xanthine-guamine phosphoribosyl transferase gene (RSV gpt), as an internal control. The gpt activity was determined by a newly developed rapid sensitive assay method. Normalization of transfection efficiency of each plate by the gpt activity permitted much more reliable comparison of different CAT constructs.

Using this quantitative CAT assay system, we have identified two positive regulatory elements, from -612 to -360 and from -111 to -72 relative to the major cap site for the DHFR transcripts. The upstream element was shown to be able to activate the DHFR minimal promoter in either orientation. In this region, there are three GC boxes that are thought to be a consensus sequence for binding of cellular activation factor, Sp 1.

The expression of the DHFR gene has been shown to be regulated during the cell cycle. To study the mechanism of this regulation, we

have measured transcriptional rate in various regions of the DHFR gene. A nuclear runoff assay showed that exon 1 is transcribed in 10 fold greater quantities than exon 2 in non synchronized cells. Our previous studies showed that 5' end region including exon 1 sequence is exposed on nucleosome free region, while the rest of the gene, down stream from exon 2, is packaged into inactive chromatin structure. These findings suggest that RNA polymerase molecule could always bind to the promoter and initiate the transcription, but the elongation of transcription is blocked within intron 1 sequence. Perhaps, during DNA replication in S phase, the inactive chromatin structure is temporarily disrupted, making the rest of the gene available for interaction with RNA polymerase. Therefore, the expression of the DHFR gene appear to be regulated at the level of elongation rather than at the level of initiation.

Proposed Course:

Our major interest has focused on the mechanism of the cell cycle regulation of the DHFR gene. We are attempting to synchronize tissue culture cells with amplified DHFR genes. Using the cells at particular cell cycle stage, we want to seek changes in promoter activity, transcription pattern and chromatin structure during the cell cycle. We are also planning to develop a new methotrexate resistant DHFR minigene. We have constructed a new DHFR minigene driven by the SV40 promoter enhancer unit. Various point mutations will be introduced into the new minigene by site directed mutagenesis. A modified DHFR minigene which produces an enzyme having a low affinity for folic acid would be an ideal in viro selectable marker for gene therapy protocols.

Publications:

1. Shimada, T. A rapid and sensitive assay system for bacterial gpt activity in transfected mammalian cells. Nucl. Acids Res. 15:4992, 1987.

2. Shimada, T., Inokuchi, K., and Nienhuis, A.W. Site specific demethylation and normal chromatin structure of the human DHFR gene promoter after transfection into CHO cells. Mol. Cell. Biol. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02313 05 CHB

PERIOD COVERED
October 1, 1986 - September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Regulation of Hemoglobin Switching During Development: Characterization of Globin Gene Promoters

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

PI: Henry J. Lin, M.D.
Others: Nicholas P. Anagnou, M.D., Guest Worker, CHB, NHLBI
Tim Rutherford, Ph.D., Visiting Associate, CHB, NHLBI
Austine Moulton, Research Assistant, CHB, NHLBI
Amanda Cline, Research Assistant, CHB, NHLBI
Arthur W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Hematology

SECTION

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National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

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☐ (a1) Minors
☐ (a2) Interviews

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

Our aim is to understand mechanisms involved in globin gene regulation, particularly developmental switching. To study regulatory sequences of the globin gene promoter and flanking region, we constructed a series of composite promoters, each containing a fragment from the fetal $\gamma$-globin upstream flanking region joined to a $\beta$-globin promoter. Their function was assessed in stably transformed human K562 cells, an erythroid cell line that expresses the $\gamma$-globin gene but not the $\beta$. A $\gamma$ fragment spanning positions -259 and -137 activated the nonfunctional $\beta$ promoter in these cells. Further analysis of this 120 bp sequence may identify elements controlling the switching of the fetal $\gamma$-globin gene.

PROJECT DESCRIPTION

Objectives:

The globin genes show three types of specificity: developmental, tissue, and maturational. Developmental specificity is shown by the orderly switching of the different globin genes during ontogeny. Tissue specificity is exhibited by their expression in only erythroid cells. Maturational specificity is shown by the increase in the proportion of globin messenger RNA during erythroid cell maturation leading to nearly exclusive globin synthesis in reticulocytes. Our object is to define the sequences within the promoter and flanking region important to the developmental specificity of the fetal $\gamma$-globin gene. Understanding this aspect of globin gene regulation is important clinically, because activation of the dormant fetal globin gene is a potential approach to the treatment of patients with severe $\beta$-thalassemia or sickle cell anemia. We showed earlier that a 270 bp fragment from the 5' flanking region of the fetal $\gamma$-globin gene is involved in the regulation of the gene. This report describes further analysis of the $\gamma$ upstream region.

Methods:

We used K562 cells to study developmental switching. These cells produce embryonic and fetal globin but not adult $\beta$-globin. We constructed $\gamma/\beta$ promoters consisting of a DNA fragment from the $\gamma$-globin gene 5' flanking region joined to a $\beta$ promoter fragment that is essential for $\beta$-globin gene expression in other test cells. Function of the $\gamma/\beta$ promoters in K562 cells would be interpreted as activation of the $\beta$ promoter by the $\gamma$ fragment.

For convenience, the composite $\gamma/\beta$ globin promoters were joined to the neomycin resistance ($neo^R$) gene, as if the $neo^R$ gene were a globin gene. The gene, carried in a recombinant plasmid vector, was introduced into K562 cells by electroporation. The cells were selected with the neomycin analog, G418. Promoter function was measured by the frequency of G418-resistant colonies that formed. The composite $\gamma/\beta$ promoters were compared to intact $\beta$ and $\gamma$ promoters.

Major findings:

A $\beta$ promoter alone only weakly drove the $neo^R$ gene. In contrast, a $\gamma$ promoter resulted in strong expression of the $neo^R$ gene, as did composite $\gamma/\beta$ promoters. When the length of the $\gamma$ portion of a composite promoter was trimmed to 120 bp by restriction enzymes, expression of the $neo^R$ gene was not reduced. An activating element appeared to lie between positions -259 and -137 of the fetal $\gamma$-globin 5' flanking region.

Proposed Course of the Project:

We have produced a set of composite $\gamma/\beta$ promoters containing different fragments of the putative 120 bp upstream activating region. Tests of these promoters should help identify critical control regions. The information obtained may be useful for developing strategies for purification of regulatory factors that bind to the upstream region.

Publications:

1. Rutherford, T., Nienhuis, A.W.: Human globin gene promoter sequences are sufficient for specific expression of a hybrid gene transfected into tissue culture cells. Mol. Cell. Biol. 7:398-402, 1987.

2. Lin, H.J., Anagnou, N.P., Rutherford, T.R., Shimada, T., Nienhuis, A.W.: Activation of the human $\beta$-globin promoter in K562 cells by DNA sequences 5′ to the fetal $\gamma$- or embryonic $\zeta$-globin genes. J. Clin. Invest. 80:in press, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 HL 02314 05 CHB

PERIOD COVERED

October 1, 1986-September 30, 1987

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)


PI: Philip J. Browning, M.D., Guest Worker

Others: A. W. Nienhuis, M.D., Branch Chief, CHB, NHLBI

E. Feingold, Ph.D., Guest Worker


COOPERATING UNITS (if any)

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SECTION

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☐ (a1) Minors

☐ (a2) Interviews

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


The c-fms gene encodes a transmembrane protein with tyrosine kinase activity that serves as the receptor for the monocyte-macrophage growth factor, CSF-1. The retroviral homologue of c-fms, v-fms, is the transforming gene of the Susan McDonough strain of the feline sarcoma virus. Using the virus as a model, we have been attempting to identify those alterations in the c-fms gene which create a transforming gene. The carboxyl terminal 40 amino acids of the c-fms gene product are replaced in v-fms by 11 amino acids encoded by the retroviral genome. A number of hybrid genes consisting of the ligand binding and transmembrane domains of the v-fms gene, and the tyrosine kinase domain and C-terminus of either v-fms or c-fms were created and the transforming potential of their gene products were assayed in an NIH 3T3 focus assay. One gene, which contained the v-fms C-terminus had equivalent transforming ability as the normal v-fms. In contrast, those genes containing the c-fms C-terminal sequences displayed a marked reduction in transformation. Mutation of the tyrosine at position 969 (the penultimate amino acid of the c-fms molecule) to a phenylalanine in a non-transforming hybrid gene creates a moderately transforming molecule. Truncation of a non-transforming hybrid gene containing the complete c-fms internal domain at the point where v-fms normally terminates renders the gene product transforming. The results of these experiments strongly suggest that the C-terminal sequences play a role in determining the transforming potential of the fms gene. Specifically, the c-fms C-terminal sequences appear to act in the negative regulation of the gene product since mutation or deletion of these sequences results in the expression of a transforming protein.

Project Description

Objectives:

The receptor for the monocyte-macrophage specific growth factor, CSF-1, is encoded by the proto-oncogene c-fms. This 165 kilodalton transmembrane glycoprotein is a tyrosine kinase as is its viral homologue, v-fms, the transforming gene of the Susan McDonough strain of feline sarcoma virus (SM-FeSV). Comparison of the human c-fms and v-fms genes revealed that the v-fms gene product is truncated at the carboxyl (C-) terminus and that 40 amino acids at the C-terminus of the c-fms gene product are replaced in v-fms by 11 amino acids encoded by the retroviral genome. The tyrosine kinases are 95% homologous at the amino acid level. We had previously shown that hybrid v-fms/c-fms genes, in which either the entire cytoplasmic domain or the C-terminal coding sequences of the v-fms gene were replaced by the analogous parts of the c-fms gene, had reduced abilities to transform fibroblasts even when expressed at high levels on the surface of NIH 3T3 cells. Thus, modifications in the C-terminus of the v-fms gene product altered its capacity to induce cellular transformation.

Our main objective has been to more closely examine differences in the fms tyrosine kinase domains and C-termini in order to understand the mechanism of transformation by v-fms. Specifically, we planned to create additional hybrid v-fms/c-fms genes and to mutate specific amino acids in the c-fms tyrosine kinase domain and C-terminus where they differ from v-fms, and subsequently examine their transforming activities in the NIH 3T3 focus assay.

Methods:

To create hybrid v-fms/c-fms genes, specific restriction endonuclease fragments derived from the human c-fms cDNA were substituted for the corresponding fragments in the SM-FeSV genome.

To create specific amino acid substitutions, oligonucleotides consisting of single nucleotide mismatches at the codon of interest were synthesized and used in site-directed mutagenesis. Mutants were confirmed by the dideoxy method of DNA sequencing.

Transformation was assayed by quantitation of focus formation. Specific recombinant DNA molecules bearing the hybrid and/or site-specifically mutated genes were introduced into NIH 3T3 cells by the calcium-phosphate co-precipitation technique. Subsequently, these cells were carried in culture for 3-4 weeks, fixed using a formaldehyde/methanol solution and foci identified by giemsa staining.

Major Findings:

A v-fms/c-fms hybrid gene was created in which the entire tyrosine kinase domain the v-fms was replaced with that of c-fms, leaving the amino and carboxyl termini from v-fms (v/c/v). In an NIH 3T3 focus assay, this hybrid gene formed foci at a similar efficiency to v-fms. This result is in sharp contrast to the absent transforming capabilities of the hybrid gene in which the entire internal domain of v-fms was replaced with

sequences from c-fms and provides further evidence that differences in the amino acid residues at the C-terminus may be involved in determining the transforming ability of the fms genes. Further, in collaboration with Drs. Morrison and Roberts of the Dana Farber Cancer Institutes in Boston, Massachussetts, the *in vivo* tyrosine phosphorylation of the fms glycoprotein was analyzed in cell lines expressing the hybrid genes using an anti-phosphotyrosine antibody. No phosphotyrosine was detected in cells expressing a non-transforming hybrid gene, whereas phosphotyrosine residues were detected in cells expressing the transforming v/c/v hybrid gene as well as cells expressing the normal v-fms gene. Thus, phosphotyrosine residues were detected only in those fms molecules that were transforming and may play a role in the transformation process.

Mutation of the tyrosine at position 969 (the penultimate amino acid of the c-fms molecule) to a phenylalanine in the non-transforming hybrid gene in which only the C-terminus of v-fms is replaced with c-fms creates a moderately transforming molecule. This tyrosine residue may therefore function in the negative regulation of tyrosine kinase activity, as has been hypothesized for c-src.

Using the non-transforming hybrid gene in which the entire v-fms internal domain was replaced with that of c-fms, two truncation mutants were made at the C-terminus. One is located at the site where the v-fms and c-fms sequences radically diverge and the second includes 11 additional amino acids, corresponding to the number of amino acids present in the normal v-fms gene product. Both of these mutations resulted in the expression of a moderately transforming protein as determined by the NIH 3T3 focus assay. This further suggests that the C-terminal sequences are involved in the normal regulation of the fms gene product and when deleted, give rise to a transforming protein.

Significance to biomedical research and the program of the institute.:

The c-fms gene (CSF-1 receptor) is expressed on the cell surface of progenitor and mature cells of the monocyte-macrophage lineage. The ability of these cells to respond to CSF-1 through the receptor is required for maintenance of cell viability, cell proliferation and differentiation. This gene, along with other genes that are known to play a role in hematopoiesis (PDGF-R, CSF-1, GM-CSF, and IL-3) is deleted in the 5q- syndrome, a transfusion-dependant refractory anemia in which 10-20% of the patients develop acute leukemia. Mutations in the remaining allele may be a cause of the resultant leukemia. A detailed understanding of alterations in c-fms that can give rise to a transforming protein may provide insight into the potential of this gene to cause neoplasia.

Proposed course of this project:

We plan to further study the transforming mutations defined in the hybrid genes using the intact mouse c-fms gene. Similar studies were not possible since we did not have the entire human c-fms cDNA. Currently, we believe that we have cloned a full length mouse c-fms cDNA, although nucleotide sequencing will be necessary to confirm this.

We also plan to create a chimeric receptor using the c-fms internal domain and the epidermal growth factor (EGF) receptor transmembrane and ligand binding domains. We have chosen the EGF receptor because EGF has no known affect on normal hematopoeisis. Once we have established that this receptor is functional, we will infect bone marrow cells and examine the proliferative response of various cell lineages to EGF. Ultimately, we wish to select certain cell populations that have a growth advantage provided by EGF. We will also study the role of transforming mutations defined above on cell transformation in the presence and absence of EGF.

Finally, we plan to study the structure of the c-fms gene in those neoplasms that contain cells expressing c-fms to determine if similar transforming mutations occur *in vivo*.

Publications:

1. Browning, P.J., Bunn, H.F., Cline, A., Shuman, M. and Nienhuis, A.W. (1986) "Replacement of COOH-terminal truncation of v-fms with c-fms sequences markedly reduces transformation potential. Proc. Natl. Acad. Sci. U.S.A. 83:7800-7804.

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# NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02315 05 CHB

PERIOD COVERED
October 1, 1986-September 30, 1987

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 personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Neal S. Young, M.D. Section Chief, CHB, NHLBI
Others: L. Platanias, M.D., Visiting Fellow, CHB, NHLBI
E. Leonard, M.D., Guest Worker, CHB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Hematology

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

| TOTAL MAN-YEARS: | PROFESSIONAL: | OTHER: |
|---|---|---|
| 2.5 | 2.5 | 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.)

Laboratory and clinical studies have suggested that aplastic anemia may be immunologically mediated. In a multi- center trial, over 150 patients with aplastic anemia and a variety of hematologic failure syndromes entered a controlled trial of anti-thymocyte globulin (ATG, Upjohn). Approximately 50% of patients with acute severe aplastic anemia had hematologic improvement, usually to transfusion independence, within three months. There were no significant differences between 10 and 28 days of therapy. Patients with chronic severe and moderate aplastic anemia also responded to 10 days of ATG; patients with pancytopenia and cellular bone marrow behaved similarly. However, other hematologic disorders including myelofibrosis, paroxysmal nocturnal hemoglobinuria, pure red cell aplasia, and amegakaryocytic thrombocytopenia, did not respond to ATG. We have also treated patients with cyclosporin A, an agent with more specific effects on T-cell function. Fifteen patients with severe aplastic anemia who had failed ATG therapy were treated with cyclosporin, for 3 months without and then for 3 months with prednisone. Five responded. All patients recovered during the time of combined cyclosporin and corticosteroid therapy, and there have been no relapses. Five patients who received shorter courses of cyclosporin A prior to ATG did not respond. No patients with Diamond- Blackfan syndrome recovered with cyclosporin A, but one of two patients with adult pure red cell aplasia had a complete hematologic remission. Cyclosporin A as well as ATG appears to be effective therapy in aplastic anemia. Finally, preliminary data has suggested a fundamental immunological defect in aplastic anemia: interleukin I (Il-1) production by monocytes from patients with aplastic anemia is markedly decreased, and, conversely, Il-1 production in patients with myelofibrosis, a hyperproliferative disorder, is markedly increased.

Project description:

Objectives:

ATG and ALG, serum preparations against human thymocytes and lymphocytes respectively, have had activity in aplastic anemia in large uncontrolled trials in Europe (ALG) and small controlled trials in the United States (ATG). The mechanism of action of ATG is unknown, and it may be immunosuppressive, stimulatory of hematopoietic growth factor production, or directly active on hematopoietic stem cells. We have implicated a population of activated suppressor lymphocytes (Leu $2^+$, $HLADR^+$, $Tac^+$) in the circulation of patients with aplastic anemia that produce inhibitory lymphokines as pathogenic in this disease, and ATG therapy consistently decreased this population, to normal levels in responding patients. From the response of patients with aplastic anemia to ATG, an immunological mechanism has been inferred. However, because of ATG's nonspecific binding to human cells and diverse potential mechanisms of action, the success of more specific therapy would be more informative of an underlying mechanism. Cyclosporin A, commonly used in transplantation as an immunosuppressive agent, has specific activity on the proliferation of T cells, probably by blocking interleukin II production.

We have previously defined a variety of immunological abnormalities in patients with aplastic anemia, including the population of activated suppressive lymphocytes described above, increased production by lymphocytes *in vitro* of gamma interferon and interleukin II, circulating gamma interferon in a minority of patients, and inverted helper/suppressor ratio of peripheral blood T-lymphocytes, and depressed natural killer cell function. Among the immune mediators studied, only interleukin I production by monocytes was found depressed in patients with aplastic anemia. Interleukin I, a molecule with a central role in the immune system, has recently also been described as having actions on hematopoietic cells, both direct and indirect. Interleukin I is equivalent to hematopoietin I, a molecule which acts syntergistically with interleukin III to promote cycling of hematopoietic stem cells. Hematopoietin I is the only factor known to act only on hematopoietic stem cells, without activity on more mature cells. In addition, interleukin I stimulates endothelial cells to produce GM-CSF and presumably other hematopoietic growth factors. Decreased interleukin I production by monocytes from aplastic anemia patients would therefore be one mechanism by which stem cells would remain quiescent and not produce progeny. We have also measured increased levels of interleukin I production by monocytes from patients with myelofibrosis. Myelofibrosis is distinguished by very high numbers of circulating hematopoietic progenitors, consistent with fundamental pathogenic role for interleukin I production in bone marrow disease.

Methods:

ATG therapy was tested in patients with aplastic anemia (acute severe and either chronic severe or moderate) as well as a variety of other hematologic disorders in a multicenter trial that enrolled over 150 patients. The trial was directed by this branch, with randomization and data collection centered at NIH. Cyclosporin A was tested in a single institution trial in patients with refractory severe aplastic anemia; some patients with aplastic anemia were treated prior to ATG at a time of ATG

unavailability. In addition, patients with adult and congenital forms of pure red cell aplasia also received cyclosporin A. Cyclosporin A levels were monitored by radioimmunoassay and adjusted for increased abnormality of liver function studies or creatomome as well as for symptoms. Data have been analyzed by standard statistical methods using the SAS Program.

Interleukin I has been measured by biological assay using Il-1 responsive mouse thymocyteo. More recently, interleukin I has been measured by an Elisa assay and interleukin I mRNA has been sought by in situ hybridization of bone marrow biopsy specimens using an Il-1 specific DNA probe.

Results:

Multi-center trial of ATG in a bone marrow.

The results of the multi-center trial are summarized in the appended tables. Results have been analyzed by three methods. First, blood counts pre-and-post treatment have been quantitatively assessed: granulocyte number has proven to be most useful because of lack of interference by transfusions. Reticulocytes, because of large variance of error, have not been useful in determining a response to treatment. Second, the principal investigators have judged whether the patients were independent of a need for transfusion at the completion of therapy. This method has the disadvantage of understating the value of an intervention in raising the granulocyte number, which may be life saving in some patients with severe bone marrow failure. Finally, the data have been analyzed by blinded observers for clinically significant improvement: patients were judged to have either restoration of completely normal blood counts, improved blood counts with transfusion independence, clinically significant improvement in blood counts (for example, meaningful improvements in granulocyte number to levels greater than $500/mm^2$), improvement in blood counts that were not clinically significant, no change in blood counts, or death.

Group I consisted of patients with severe aplastic anemia who were randomized to receive either 10 days or 28 days of therapy. There were no significant differences in outcome by any criteria between these two regimens, although there was a trend towards higher blood counts and higher response rate in the patients who received larger amounts of ATG. Group II consisted of patients with either chronic severe aplastic anemia or moderate aplastic anemia; because of the better prognosis of this group compared to acute aplastic anemia, randomization to conventionl therapy, consisting of high dose androgens, was ethically justified. There was no response rate to Deca-durabolin. However, there was a significant response rate of patients in this category to ATG, although somewhat lower, there was no statistically significant difference in the response rate of patients with chronic severe aplastic anemia who received 10 days of ATG and patients with acute severe disease treated similarly. Finally, a heterogeneous group of disorders constituted group III. Surprisingly, patients with pancytopenia and cellular bone marrow appeared to respond to ATG with a similar rate to patients with chronic aplastic anemia. However, patients with more limited forms of single lineage bone marrow failure did not respond to ATG. The data are summarized in the following tables:

ACUTE SEVERE APLASTIC ANEMIA (GROUP I)
Clinical Results by Consensus

OUTCOME

| | normal blood counts | tx-ind | significant improvement | increased blood counts | no change | death |
|---|---|---|---|---|---|---|
| 10 days N=41 | 1 (2%) | 7 (17) | 10 (24) | 4 (10) | 9 (22) | 10 (24) |
| | | 44% | | | | |
| 28 days N=36 | 3 (8) | 6 (17) | 9 (25) | 3 (8) | 4 (11) | 11 (31) |
| | | 50% | | | | |

CHRONIC SEVERE APLASTIC ANEMIA (GROUP II)
Clinical Results by Consensus

OUTCOME

| | normal blood counts | tx-ind | significant improvement | increased blood counts | no change | death |
|---|---|---|---|---|---|---|
| ATG 10 days N=17 | 1 (6%) | 1 (6) | 3 (18) | 0 | 11 (65) | 1 (6) |
| | | 29% | | | | |
| Deca-durabolin N=10 | 0 | 0 | 0 | 1 (10) | 8 (90) | 1 (10) |
| | | 0 | | | | |

CHRONIC SEVERE AND MODERATE APLASTIC ANEMIA (GROUP II)
Clinical Results by Consensus

OUTCOME

| | normal blood counts | tx-ind | significant improvement | increased blood counts | no change | death |
|---|---|---|---|---|---|---|
| ATG N=25 | 1 (4%) | 2 (8) | 4 (16) | 0 | 16 (64) | 2 (8) |
| | | 25% | | | | |
| Deca-durabolin | 0 | 0 | 0 | 3 (16) | 14 (74) | 2 (11) |
| | | 0% | | | | |

Cyclosporin A in Aplastic anemia and pure red cell aplasia.
Cyclosporin A was administered to maintain blood levels between 200-400 ng/ml. In some patients, hematologic improvement was correlated with cyclosporin A levels. A response rate of approximately 33% was observed in patients who had been refractory to ATG (see appended table). Almost all patients responded during the period of concomitant treatment with corticosteroids. Patients treated for briefer periods of time prior to ATG therapy did not show significant response rate, although 3/5 patients subsequently responded with good remissions to ATG. One patient with adult pure red cell aplasia sustained a complete remission on cyclosporin A therapy, but the treatment was not effective in patients with Diamond-Blackfan syndrome. Remissions have been sustained for 3-9 months in patients with aplastic anemia who responded to cyclosporin A. Toxicity was mainly renal and always reversible with dose reduction. The data are summarized in the table below:

Cyclosporine Protocol 8/6/87

| | #enrolled | #responders $\bar{p}$ 3 mos rx | #responders $\bar{p}$ 6 mos rx | total # responders |
|---|---|---|---|---|
| Aplastic Anemia | 15 | 1/11 | 4/8 | 5 |
| Diamond-Blackfan | 8 | 0/7 | 1/6 | 1 |
| PRCA | 2 | 1/2 | 1/2 | 1 |

| | #enrolled | #responders | Duration rx |
|---|---|---|---|
| Acute Aplastic Anemia | 5 | 0 | 1-5 mos |

Interleukin I Production.

Interleukin I, as measured in a bioassay, was underproduced by monocytes of eleven patients with aplastic anemia. In contrast, five patients with myelofibrosis demonstrated mark overproduction of interleukin I. These data suggest a possible regulatory role for this molecule in hematopoietic stem cell cycling and progenitor production.

Future Course For Projects.

Clinically, cyclosporin A will be combined with ATG and the response rate in a group of patients defined to combined immuno-therapy. In addition, this branch will conduct a trial of GM-CSF, a human hematopoietic growth factor, in patients with aplastic anemia prior to ATG. Interleukin I studies will be extended to analysis of interleukin I transcription in bone marrow cells of aplastic anemia and myelofibrosis patients and measurement of interleukin I in the serum by Elisa assay.

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3. Bielory, L., Lawley, T., Gascon, P., Yancey, K., Young, N., and Frank, M.M.: Human serum sickness. Medicine. In press, 1987.

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5. Ettinghausen, S.B., Moore, J.G., Platanias, L., Young, N., and Rosenberg, S.A.: Hematologic toxicity of lymphokine activated killer (LAK) cells and recombinant interleukin 2 in cancer patients. Blood. 69:1654-1660, 1987.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02319 04 CHB

PERIOD COVERED
October 1, 1986-September 30, 1987

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
B19 (Human) Parvovirus

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

PI: Neal Young, M.D.

Others: K. Ozawa, M.D., Visiting Fellow, CHB, NHLBI
G. Kurtzman, M.D., Medical Staff Fellow, CHB, NHLBI
L. Platanias, M.D., Visiting Fellow, CHB, NHLBI
S. Kachigaya, M.D., Visiting Fellow, CHB, NHLBI
J. Ayub, M.D., Chemist, CHB, NHLBI
Y.-S. Hao, M.D., Visiting Fellow, CHB, NHLBI
T. Shimada, M.D., Visiting Fellow, CHB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Hematology

SECTION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20892

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CHECK APPROPRIATE BOX(ES)

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☐ (a1) Minors

☐ (a2) Interviews

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


Studies of the B19 parvovirus have utilized a suspension culture system for propogation of the virus for detailed molecular analysis of virus replication, transcription, and protein synthesis. B19 parvovirus DNA replication is similar to that of other parvoviruses, proceeding through double-stranded high molecular weight intermediate forms. B19 parvovirus proteins are also similar to those of other parvoviruses, consisting of two capsid proteins of 58 and 83 kd and at least one nonstructural protein of 77 kd. In contrast to other parvoviruses, the transcription map of the B19 parvovirus is extraordinarily complex, and detailed analysis has shown that regulation, independent of promoter strength, likely results from differential splicing or transcription termination. Specific virus protein products have been assigned to RNA species, and viral proteins translated in vitro. A cytotoxic function has been assigned to the nonstructural protein using transfection of recombinant constructs into HeLa cells. In clinical studies, the spectrum of parvovirus disease has been expanded by the description of the first case of persistent B19 infection, in a child with congenital immunodeficiency, result in chronic bone marrow failure. The normal host defense to B19 parvovirus infection appears to be predominantly humoral and mediated by neutralizing antibodies. B19 parvovirus has been detected in the circulating cells of patients acutely infected and may be present in granulocyte precursors. In parallel studies of feline panleukopenia virus, a common natural cause of neutropenia and aplastic anemia in cats, virus replication and propagation have been detected in vitro using bone marrow cultures and shown to be dependent upon the addition of hematopoietic colony stimulating factors. Feline panleukopenia virus is a potent inhibitor of colony formation by cat progenitor cells from all lineages.

classical S1 analysis, RNA run-off experiments, and RNAse protection assays. Virus proteins have been analyzed by immunoprecipitation with specific antisera and Western blot analysis. Clinical samples have been subjected to DNA dot blot and Southern gel analysis, and antibody specificity has been analyzed by immunoblotting. B19 parvovirus proteins have been synthesized in vitro with synthetic RNA templates and a cell free translation system. Parvovirus has been labeled in vitro by $^{125}I$-iodination and metabolically in suspension cultures by addition of $^{35}S$-methionine or $^{3}H$-amino acids for binding studies. Analysis of protein function has been performed using transfection of constructed plasmas into HeLa and other cell types and measurement of antibiotic-resistant colony formation in the presence of G418.

Complementary studies at the molecular level have been performed with feline panleukemia virus using appropriate probes and antibodies.

Major Findings:

A tissue culture system for B19 parvovirus propogation.: Inoculation of human bone marrow cells in suspension cultures with B19 parvovirus results in a marked decline in the number of identifiable erythroid cells over the course of 7-9 days of incubation. Characteristic large early erythroid cells ("giant pronormoblasts") are observed on Wright's-Giemsa stain of infected cultures at 48 hours, similar to the cells observed in the bone marrow of patients with transient aplastic crisis. By in situ hybridization, 30-40% of erythroblasts are infected at 48 hours; a similar proportion of cells show evidence of B19 capsid protein by immunofluorescence. B19 DNA can be detected by dot blot only in the erythroid and not in the leuocyte fraction of bone marrow cells, separated by an immune panning method. B19 replication, as determined by Southern analysis, and B19 encapsidation, determined by sensitivity of isolated cell fractions to DNAse I, are restricted to the nucleus. The time course of B19 parvovirus infection is similar to that described for other parvoviruses in tissue culture: B19 DNA is detectable in the nuclei of infected cells at 18 hours and in the supernatants at 32 hours; B19 genome copy number is approximately 25-30,000/infected cells at 48 hours. Recovery of virus is highly dependent upon the multiplicity of infection, with as much as 200-fold the input virus recovered from total cultures and 50-fold from culture supernatants. Virus released into the supernatant of erythroid bone marrow cultures is more infectious than virus obtained from sera of infected patients. This culture system not only mimics the natural course of parvovirus infection but represents a safe and convenient method for the study of the molecular biology of this human pathogenic parvovirus.

Novel transcription map for B19 parvovirus. Using this culture system, a transcription map for B19 parvovirus has been developed. While replication of B19 viral DNA and the viral proteins produced by B19 parvovirus infection are similar to those of other animal parvoviruses, the pattern of RNA transcription is different from both the autonomous and adenoassociated parvovirus genera. There are nine overlapping polyadenylated transcripts in infected cells, all but one of which contains large introns. In addition to the large number of transcripts, B19 differs from other parvoviruses in initiation of all transcription from a single strong left side promoter; there is no internal promoter as found in other Parvoviridae. Leader sequences of about 60 bases are utilized to drive transcription of the middle and right side transcripts from the P6 promoter. There are two separate transcription termination sites, in contrast to the single far right side termination site of other parvoviruses, and

utilization by three transcripts of a variant polyadenylation signal in the middle of the genome. Two transcripts from the middle of the genome are particularly abundant, but there is no evidence that they encode proteins. By a number of methods, including negative hybrid selection, the specific protein products of B19 were correlated to the predicted transcripts. The left side encoded at least one large nonstructural protein of approximately 77 kd, which was localized to the nucleus. The VP1 capsid protein of 84 kd was the minor structural species, and the major capsid protein (VP2) was 58 kd. The transcription map of B19 parvovirus suggests that it represents a distinct parvovirus, markedly different from both the autonomous and dependent parvoviruses. In addition, the absence of multiple promoters indicates that regulation of transcript and protein abundance must occur by a mechanism other than diffential promoter strength, likely pausing or termination of RNA transcription or regulation through splicing.

Function of the nonstructural protein.: The nonstructural protein of the B19 parvovirus may be cytotoxic. Experiments were performed in which the nonstructural gene (left side of the B19 genome) was transfected into HeLa cells in the presence of the neomycin resistance gene. Under circumstances in which the B19 nonstructural gene would be predicted to function, no antibiotic-resistant colonies were obtained. In contrast, sequences from the middle or right side of the B19 genome did not interfere with transformation. These results suggest that the nonstructural gene may encode for a protein that blocks cell proliferation or causes cell death. In particular, this effect is independent of cell type and may represent a mechanism of inhibition of myelopoiesis that results in clinical neutropenia in infected patients.

Chronic bone marrow failure due to persistent B19 parvovirus infection.: A child with Nezelof's syndrome, a congenital immunodeficiency disease, abruptly developed anemia and neutropenia at the time of seroconversion to B19 parvovirus positivity by both DNA and antigen assays. Parvovirus has been present in this child's serum intermittently for over one year, with titers inversely correlated to hemoglobin and neutrophil count. Parvovirus has been demonstrated in the patient's bone marrow by immunofluorescence and in situ hybridization. Southern gel analysis has demonstrated replicative forms restricted to the bone marrow. Persistence of B19 parvovirus infection in this individual is almost certainly the result of a defect in production of specific antibody to capsid protein. Although IgG and IgM directed against the parvovirus are present by radio-immunoassay, Western blot failed to demonstrate antibodies in the patient's sera that bound to either of the capsid proteins. A cellular defect cannot be excluded, but no evidence of lymphocyte activation by parvovirus antigen has been found even in individuals who have had a history of parvovirus infection and are seropositive.

Feline panleukopenia virus. Feline panleukopenia virus inhibits cat hematopoiesis in viro and in vitro. Cultures of feline bone marrow and lymphocytes have been inoculated with feline panleukopenia virus, quantitatively measured in a radioplaque assay. This virus proliferates in bone marrow, and replication is increased by the addition of colony stimulating factor and erythroproietin. In lymphocytes, replication also occurs but is responsive to protein A, a cat mitogen. Feline panleukopenia virus proteins can be demonstrated by immunofluorescence, RNA/DNA by in situ to hybridization, and replicative intermediate forms by Southern gel analysis of cells from infected bone marrow cultures. In contrast to B19 parvovirus, feline panleukopenia virus

is a potent inhibitor not only of erythroid but also of myeloid colony formation, consistent with its generalized effects on cat hematopoiesis.

Proposed course of Project:

It seems likely that erythroid specificity may be linked to the unusual transcription map of the B19 parvovirus. Preliminary data has suggested that in nonpermissive cells--Hela, 3T3, and human granulocytes--some transcription occurs but is restricted to the left side of the genome. This would support a mechanism of premature termination or transcriptional pausing in the regulation of parvovirus transcripts. If true, accumulation of the nonstructural protein may result in cell death, with different kinetics and specifity than cell death due to intracellular viral propagation. B19 parvovirus infection in infected cells may represent a convenient model for the assay of RNA transcription termination factors in eukorytic cells. Further efforts will be devoted to isolation, purification, and production of sufficient quantities of nonstructural protein for investigation of the mechanism of its effects on cells, particularly to distinguish between an anti-mitotic and cytotoxic effect.

The discovery of persistent parvovirus infection in a child with immuno-deficiency significantly expands the spectrum of parvovirus disease in humans. It would be expected that parvovirus infection may result in occult bone marrow failure in others with immunodeficiency, for example following cancer chemo-therapy and in patients with acquired immunodeficiency syndrome. Sera from these patients are currently being screened for evidence of persistent viral infection.

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