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(OCT. 1, 1994 - SEPT. 30, 1995)

ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR, NHLBI
OCTOBER 1, 1994 - SEPTEMBER 30, 1995

With some additional reorganization this year, the NHLBI Division of Intramural Research now comprises 15 research Laboratories and Branches, one independent Section and one service Laboratory supporting animal research. The research is directed by approximately 60 independent tenured and tenure-track investigators and the total research staff, including technicians, postdoctoral fellows (315) and guest workers (85), is numbers approximately 600 with an additional 75 students participating during the summer months. The scientific staff is supported by an administrative staff of 8 and 12 purchasing agents. Additional support is provided by the budget and personnel offices and a computer-support group working out of the office of the Director, NHLBI. This annual report describes the research conducted by intramural scientists during fiscal year 1995 - October 1, 1994 to September 30, 1995. Throughout the Division, there is a central theme of studies devoted to understanding the fundamental biological processes of differentiation and development, cell structure and function,and tissue organization and function at the molecular and genetic level; and understanding the molecular basis of cardiovascular, blood and pulmonary disease in order to develop molecular and genetic therapies. The activities of each of the Laboratories and Branches is described briefly below. In subsequent pages, the Laboratory and Branch Chiefs have summarized the major accomplishments in their areas during the past year, and detailed progress reports on each project follow these summaries.

The **Pathology Section** (Dr, Victor Ferrans, Chief) has conducted morphological and cytological studies in the following areas of research: identification of inflammatory cell types in interleukin-2-induced myocarditis; acute and chronic myocarditis associate with Chagas' disease; cardiomyopathy due to deposits of amyloid material and histiocytoid cardiomyopathy; apoptosis in the cardiovascular system; cardiac differentiation induced by triiodothyronine; evaluation of the basis of mitral valva allograft failure in a sheep model; mitral valve floppiness in patients with idiopathic mitral valve prolapse; and, in collaboration with the Pulmonary-Critical Care Medicine Branch, studies of pulmonary lesions experimentally induced by silicosis and bleomycin.

The research of the **Cardiology Branch** (Dr. Stephen Epstein, Chief) is described under 5 major categories. *Molecular vascular biology* studies include viruses and viral-mediated mechanisms as contributors to the development of restenosis and atherosclerosis (in particular cytomegalovirus), adenoviral-mediated gene transfer for the potential treatment of restenosis, and signal transduction pathways in vascular smooth muscle cells. Studies on the *enhancement of coronary collateral development* include the use of angiogenic peptides in dogs and a Phase I study in patients, and a gene therapy approach utilizing adenoviral-mediated gene transfer of growth factors. Studies on the multiple genetic causes of the inherited cardiac disease *hypertrophic cardiomyopathy* continue as well as the development of novel therapeutic strategies following the previous success of

DDD pacing. The role of in *endothelium in vascular tone, platelet function and microvascular dysfunction* continues to be a major focus. Studies include: endothelial function in hypertension and hypercholesterolemia; regulation of microvascular function in the human coronary and systemic circulation; relation between coronary artery disease risk factors and endothelial dysfunction. Current research on *coronary artery disease and heart failure* includes the biochemical detection (uptake of ^{18}F-deoxyglucose) of viable myocardium; congestive heart failure, specifically comparison of myocardial fibrosis in patients with ischemic an nonischemic cardiomyopathy; histopathology of explanted hearts of patients receiving heart transplants; and transesophogeal dobutamine stress echocardiography in patients with coronary artery disease and aortic regurgitation.

The **Hematology Branch** (Dr. Neal Young, Chief) interests are in normal hematopoiesis and the pathogenesis, pathophysiology, and effective treatment of bone marrow failure and malignant blood cell disease. Disease under study include acquired aplastic anemia, Fanconi's anemia, acute and chronic myelogenous leukemia, multiple myeloma, myelodysplasia and congenital hemoglobinopathies. More specifically, work on *B19 parvovirus* includes studies of the target cell and its viral receptor (both discovered in this Branch), a phase I trial of a recombinant vaccine reagent developed as a result of work in this Branch, studies of animal parvoviruses, collaborative structural studies by x-ray crystallography and cryoelectronmicroscopy, and studies of the binding sites for other adeno-associated viruses. Investigations of causes of *bone marrow failure* include research on the molecular mechanisms for immune suppression of hematopoiesis; possible association of a novel hepatitis virus with aplastic anemia, hepatitis/anemia, and or fulminant hepatitis; the epidemiology of aplastic anemia Thailand and Vietnam; the treatment of acquired aplastic anemia by immunosuppression with ATG and cyclosporin; development of a murine cell line model for paroxysmal nocturnal hemoglobinuria; and genetic studies of Fanconi's anemia including initiation of a gene therapy protocol for Fanconi's anemia type C patients. *Gene transfer* studies in non-human primates are directed toward improving gene transduction by selection of target cells and improved and novel vectors. Studies in patients include gene transduction into hematopoietic progenitor and stem cells and initiation of a therapeutic gene therapy protocol for Gaucher's disease (in collaboration with NINDS). the major efforts in *bone marrow transplantation* research include a clinical trial of a new protocol to prevent acute graft-versus-host disease and laboratory studies to deplete marrow of the lymphocytes responsive for graft-versus-host disease while retaining the lymphocytes with graft-versus-leukemia activity.

The studies of the **Hypertension-Endocrine Branch** (Dr. Harry Keiser, Chief) on the mechanisms of regulation of blood pressure and the causes and treatment of hypertension have been concentrated in three areas. Research on *vasoactive substances* includes studies in animal models of the role of the renin-angiotensin system in congestive heart failure and the possible association of elevated levels of endothelin with the nephrotoxicity and hypertension that often accompany the use of cyclosporin A as an immunosuppressive agent to prevent allograft rejection. The mechanism of the

vasodilator activity of endothelin is also under study. Efforts continue to improve the diagnosis, localization and treatment of *pheochromocytoma.* With the appointment of a new tenure-track investigator, the Branch's research has expanded to include studies of the *molecular basis for the relationship between hypertension, insulin resistance and obesity.*

The **Molecular Disease Branch** conducts biochemical, physiological and genetic studies on plasma lipoproteins with the goals of developing new methods for diagnosis and better approaches to therapy (including gene therapy) of the genetic disorders of lipid metabolism that lead to premature cardiovascular disease and other diseases including pancreatitis. Research on *lipoprotein metabolism* includes studies in humans on the rates of synthesis and degradation of high density lipoproteins;, the extent of correlation of low levels of high density lipoproteins with premature heart disease; and the structural basis of the substrate specificities of lipoprotein lipase and hepatic lipase.

Recently investigated *genetic dyslipoproteinemias* include lecithin cholesterol acytransferase deficiency, familial hypercholesterolemia, and apoA-1 deficiency. Several *transgenic animal models of lipoprotein metabolism* have been developed. These include transgenic mice overexpressing human lecithin cholesterol acyl transferase; transgenic mice overexpressing apoprotein A-II; transgenic Watanabe rabbits (deficient in low density lipoprotein receptors) overexpressing apoA-I; and transgenic rabbits overexpressing lecithin cholesterol acyl transferase. *Gene therapy* research in mouse models includes adenoviral-mediated replacement of apoE in an apoE-deficient mouse model (knockout by homologous recombination) and adenovirus-mediated addition of human hepatic lipase in a hepatic lipase-deficient mouse strain.

Research in the **Molecular Hematology Branch** (Dr. Brian Safer, Acting Chief) is directed toward acquiring the basic knowledge and technology required for successful gee therapy. Research on *RNA and protein biosynthesis* includes studies on the mechanisms of regulation of transcription and translation. Efforts on *adeno-associated molecular biology and gene transfer* include fundamental studies on virus purification and site-specific integration as well development of AAV vectors for potential gene therapy of hemophilia B. Studies of *chromatin and gene expression* focus on the mechanism of regulation of gene expression by altered chromatin structure.

The **Pulmonary-Critical Care Medicine Branch** (Dr. Joel Moss, Chief), formed by merging the previous Pulmonary Branch with the Laboratory of Cellular Metabolism with the Cardiopulmonary Devices Section transferring from the Laboratory of Cell Biology, has initiated research on the pathophysiology of acute and chronic lung diseases utilizing the strengths of the basic scientists. Their diversified program includes the following newly initiated clinical programs: the *role of nitric oxide in the pathophysiology of lung disease* currently focussed on studies of the inducible isoform of nitric oxide synthetase which is responsible for NO synthesis in inflammatory states; and studies of the basic mechanisms and clinical consequences of *α1-antitrypsin deficiency* leading to

treatment strategies. Ongoing studies of the *regulation of vesicular trafficking by ADP-ribosylation factors* examine the function of ARFs and associated proteins including the activation of phospholipase D by ARF (in collaboration with the Laboratory of Cell Signalling). Other studies include the *regulation of ADP-ribosylation in mammalian cells* by the action of ADP-ribosyltransferases and ADP-ribosylarginine hydrolases. Studies of *cyclic nucleotide phosphodiesterases* are focussed on Type III cGMP-inhibited phosphodiesterases which are widely distributed, specifically inhibited by drugs that increase myocardial contractility and relax smooth muscle, and rapidly activated hormones such as insulin. These studies may lead to insights into airway reactivity, inflammation and allergy. Current emphasis in the *design of pulmonary and cardiac assist devices* is on methods that do not exceed normal physiological conditions keeping peak airway pressure of the mechanical ventilator and left ventricular filling pressure within the normal ranges.

The **Laboratory of Biochemical Genetics** (Dr. Marshall Nirenberg, Chief) continues to study the *NK-2 homeobox gene* in *Drosophila* which appears to code for a protein required for commitment of some cells to the neural pathway of development. A number of interesting *transgenic lines of Drosophila* have been developed and are under study and studies on previously identified *novel mouse homeobox and POU-domain genes* have been continued. The basis of the formation of *neuronal polarity in neuromuscular junction* is another area of interest as the *role of nerve-muscle adhesion in neuromuscular junction formation* and the pathway of *assembly of the presynaptic apparatus* - all by ultrastructural analysis and immunocytochemistry. Research on *apoptosis mechanisms in irradiated mouse embryo forebrain* has been initiated. Molecular and genetic studies of fundamental metabolic regulatory mechanisms continue on the highly informative bacterial sugar transport system: *phosphoenolpyruvate:sugar phosphotransferase system.* These include the structural basis of GTP binding to the phosphocarrier protein Hpr, structure-function analysis of enzyme I, chromosomal mapping of the sugar transport genes in *Mycoplasma*, and related studies of phosphate-dependent regulation of adenylyl cyclase and phosphodiesterase. Studies on the *regulation of expression of the HoxA7 gene* continue.

The **Laboratory of Biochemistry** (Dr. Boon Chock, Chief) supports a diverse program. *Biochemical studies of novel, potential regulatory reactions* include nitration of glutamine synthetase by peroxynitrate which can be formed endogenously; the increase in hydrophobicity of proteins during aging and oxidative stress; free radical-mediated changes in structure and function of glutamine synthetase, mechanisms of action of thiol-specific enzymes and reversal of oxygen-mediated damage to protein methionine groups. Major emphasis continues on studying the *biosynthesis and functions of selenium-containing biomolecules* including the mechanism of synthesis of selenophosphate, the donor of selenium to selenocysteine, the structure and distribution of the enzyme selenophosphate synthetase, and the discovery of new selenoproteins. The *formation and utilization of free radicals* was followed by EPR and spin-trapping methods. Recent studies on *regulation by reversible covalent modification of proteins* include regulation of

Ca(II) oscillations in HeLa cells and characterization of a Mg(II)-dependent, Ca(II)-inhibited phosphatase. Studies of the *physical and chemical properties of proteins of biological interest* are mostly concerned with the role of ligand binding and protein-protein and inter- and intra-subunit interactions in enzyme catalysis and regulation. Other research activities focus on the *oxidative modification of proteins* which has been implicated in important physiological and pathological processes. Last year emphasis was on identifying the most highly oxidatively modified liver proteins, glutathiolation as a reversible covalent modification of proteins, and methods for detection and quantifying oxidatively modified proteins.

The **Laboratory of Biophysical Chemistry** provides strong collaborative support to many excellent investigators in the NHLBI and NIH and also actively develops and extends physical and chemical methods as applied to biological problems. Solution and solid *nuclear magnetic resonance spectroscopy and computer modeling* are being applied to determine the structure of the homeodomain of the *Drosophila* NK-2 proteins and the active site determined by site-directed mutagenesis of protein expressed in *E. coli*. Cytochrome c is also understudy as a model of protein folding. *X-ray crystallography* has been used to determine the conformation and stereochemistry of biologically active compounds including thalidomide, anti-retroviral compounds and enzyme inhibitors. The application of novel *countercurrent chromatographic* methods previously developed in this laboratory has been extended by development of a new technique: pH zone countercurrent chromatography. The development and application of *mass spectroscopy of macromolecules* has led to improvements in the electrospray spectrometer and new cleavage reactions of proteins.

The principal goal of the **Laboratory of Cardiac Energetics** (Dr. Robert Balaban, Chief) is to understand cellular and molecular processes for the conversion of energy to useful work in order to develop new strategies for the diagnosis, prevention and treatment of heart disease. The approach is to utilize *non-invasive nuclear magnetic resonance and optical spectroscopy* to study energy metabolism, blood flow and tissue oxygenation in intact tissues and humans. This year emphasis has been on the complex interaction between *oxidative phosphorylation, muscle contraction and blood flow*. Methods are being developed and applied to monitor the NADH/AND ratio in the intact heart *in vivo*. A major effort has involved the development of new tissue imaging techniques based on the rate of magnetization transfer between protons of macromolecules and water, and for imaging low-abundance metabolites by a similar phenomenon.

Five major research activities are supported by the **Laboratory of Cell Biology** (Dr. Edward Korn, Chief). Studies on the *structure and function of unconventional non-muscle myosins* include: the localization of the active and inactive forms of the 3 myosin I isozymes in *Acanthamoeba castellanii*; further characterization of the regulation of myosin I heavy chain kinase by binding to membranes and autophosphorylation; the mechanism by which *Acanthamoeba* myosin II is regulated by phosphorylation of sites in its C-terminal tail; characterization of the phenotype of single, double and triple myosin I

mutants in *Dictyostelium discoideum*, characterization of a novel *Dictyostelium* myosin, MyoJ, which may be the first member of a new myosin family, type XI; and immunocytochemical studies of the localization and presumptive role of myosin V in melanocytes. The role of actophorin in regulating *actin polymerization* has been further defined. Studies on the *70-kDa heat shock proteins* include identification of a new cofactor, auxilin, for uncoating activity; new refinements of the kinetics of uncoating; and further characterization of he ATPase activity of hsp70. The *biological applications of florescence spectroscopy* include development of a high pressure cell and further studies of proteins-DNA, protein-ligand, and protein domain-domain interactions. *Bioenergetic* investigations have focussed on two systems: mitochondrial cytochrome *c* oxidase and bacteriorhodopsin. The lipid-dependence of the bacteriorhodopsin system and the intermediate steps of the transfer of 4 electrons from cytochrome a_3 to O_2 catalyzed by cytochrome *c* oxidase are under study.

Two major areas of research engage the **Laboratory of Cell Signaling** (Dr. Sue Goo Rhee, Chief). Studies on *signal-activated phospholipases* include: the decreased expression of phospholipase C-β2 isozyme in human platelets with impaired function; cDNA cloning; splice variants; expression and purification of phospholipase C-δ4; tyrosine kinase-independent activation of phospholipase C-γ1 by the concerted action of tau proteins and arachidonic acid; role of plekstrin homology domain in the regulation of phospholipase C-β isozymes; purification of phospholipase D and its activator from rat brain. A *Thioredoxin-dependent peroxidase* has been identified as the 25-kDa enzyme that protects proteins from oxidation systems that generate reactive oxygen and 5 homologues of the enzyme have been identified in mammalian tissues and studied.

The **Laboratory of Molecular Cardiology** (Dr. Robert Adelstein, Chief)investigates the *regulation, expression and function of contractile proteins* of smooth muscle and non-muscle cells and the *homeobox genes that function in early development* of *Drosophila* and mammalian embryos. Specific projects include: the growth and differentiation of smooth muscle and non-muscle cells; regulation of muscle and nonmuscle contractile activity by myosin light chain and heavy chain phosphorylation; neuronal-specific isoforms of myosin heavy chains; expression and study of non-muscle myosin heavy chains and mutant forms of brush border myosin I in baculovirus systems; functional studies of human cardiac myosin *in vitro* in relation to hypertrophic cardiomyopathy (in collaboration with the Cardiology Branch); overexpression of myosin isoforms in nonmuscle cells; cloning and characterization of myosin cDNAs from *Xenopus*; construction of myosin null mutants in mice by homologous recombination; regulation of myosin heavy chain gene expression in nonmuscle cells; characterization of novel nonmuscle myosins in *Drosophila*; interaction of myosin-binding proteins with smooth muscle and non-muscle myosins; the function of *Drosophila* NK-homeobox genes in mesodermal cell differentiation; function of *NK-1* homeobox gene in neuromuscular synaptogenesis.

The major efforts of the **Laboratory of Molecular Immunology** (Dr. Warren Leonard, Chief) are focussed on understanding the process of T-cell activation, understanding the

process of mast cell activation, and the mechanisms of drug-induced toxicities. Studies of *T-cell activation related to the IL-2 receptor* include further studies of the relation between mutations in the γ_c chain and X-linked severe combined immunodeficiency and the occurrence of the γ_c chain as a component of interleukin receptors; the association of IL-2Rβ and γ_c with Janus family tyrosine kinases; IL-2 signaling; the role of γ_c in lymphoid development; gene therapy for XSCID; and identification of γ_c-associated molecules. Studies on the *regulation of T-cell activation* include the regulation of the IL-2Rαc chain gene; cytokine-induced STAT systems; STAT protein activation and HTLV-1 transformation; ; and cloning human Stat5. Studies this year on the *activation of mast cells* have focussed on the roles of phospholipase D and protein kinase C; the generation of arachidonic acid and the MAP kinase/phospholipase A_2 cascade; the production of cytokines TNFα and TGFβ; and potential sites for therapeutic intervention. Investigation of the *mechanisms of pathologies caused by reactive metabolites of drugs and endogenous molecules* center on the characterization of the protein targets of reactive metabolites of inhalation anesthetic halothanes, nonsteroidal antiinflammatory agent diclofenac, the anti-hypertensive drug guanabenz, and nitric oxide. Another area of study is *neurotoxicity in dopamine-containing neurons, regulation of dopamine transport, and programmed cell death* in neuronal cultures. Also under investigation is *the role of nitric oxide in retinal function and disease.*

Analysis of normal kidney function as a basis for understanding the origins and treatment of its pathophysiology is the center of research of the **Laboratory of Kidney and Electrolyte Metabolism** (Dr. Maurice Burg, Chief). Studies in *single renal tubules* include the influence of vasopressin on the distribution of aquaporin-2 water channels and the involvement of the vesicle targeting protein VAMP-2 and syntaxins; quantification of excretion of aquaporin-2 in human urine; localization of aquaporin-3 and aquaporin-4 in rat kidney; regulation of the expression of aquaporin-2 and aquaporin-3; the dysregulation of aquaporin expression in pathophysiological processes; cloning the vasopressin-regulated urea transporter; and the inhibition of epithelial water permeability by activation of protein kinase C. Optical microscopy methods have been developed to study *transport in model epithelia* formed by culturing renal cells on glass coverslips. The studies of *organic osmolytes* that accumulate intracellularly in renal inner medulla to maintain the intracellular milieu when he interstitial fluid becomes hyperosmotic have included the mechanisms of accumulation of sorbitol from glucose by aldose reductase activity; betaine accumulation by increased transport; the accumulation of glycerolphosphorylcholine by increased cleavage of phosphatidylcholine; and he identification of other genes that respond to osmotic stress.

Annual Report of the Pathology Section
Division of Intramural Research
National Heart, Lung, and Blood Institute
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During the past 2 years the Pathology Section has undergone extensive renovation of its facilities, and this has resulted in the organization of new units for laser scanning confocal fluorescence microscopy and for morphometric studies using color video techniques. Other previously existing facilities for light and electron microscopy and video imaging have been upgraded. The renovated Section has continued to develop new research programs, with emphasis on immunohistochemical and ultrastructural aspects of cardiovascular and pulmonary diseases, and maintains strong collaborative interactions with other Branches and Laboratories of NHLBI and outside our institution. Research studies that have been brought to completion during the past year are summarized under the following categories: 1) myocarditis; 2) cardiomyopathies; 3) valvular heart disease; 4) apoptosis; 5) pulmonary differentiation; 6) pulmonary silicosis and 7) the pulmonary toxicity of bleomycin.

Myocarditis Induced By Interleukin-2

Precise identification of the types of inflammatory cells infiltrating the heart in various types of myocarditis is essential for the evaluation of these disorders. The use of immunologic markers for the histochemical typing of these cells has proven extremely useful. However, single labeling procedures do not provide information concerning the relationships that exist among different types of inflammatory cells. In order to circumvent these problems we have developed procedures for the simultaneous labeling of up to 3 different types of cells, including macrophages, interstitial dendritic cells, T-helper lymphocytes, T-suppressor lymphocytes and lymphokine-activated killer (LAK) cells. These cells can be selectively identified by their immunoreactivity with antibodies that are labeled with fluorochromes (showing blue, green and red fluorescence) in the same tissue section. As originally developed in our laboratory this method employed a fluorescence microscope with multiple bandpass filters and sequential photographs. However, after installation of the confocal microscope with 2 laser sources and the capability for simultaneously analyzing several bands of fluorescence emission, we have been able to detect and record all 3 colors in a single scan. Our first application of this technique has been for the study of the myocarditis induced by interleukin-2 in a rat model previously developed in our unit. The results obtained show that LAK cells and $CD8^+$ lymphocytes are the predominant types of inflammatory cells in this type of myocarditis, and that these cells are accompanied by numerous interstitial dendritic cells (antigen-presenting and processing cells). Additional studies, now being completed, have been made of pulmonary tissue of these animals. These studies have demonstrated a similar population profile of inflammatory cells in association with pulmonary damage induced by interleukin-2. These observations are of clinical relevance, since this damage is known to lead to a "vascular leak syndrome" in patients receiving treatment with interleukin-2 for neoplastic disorders.

Chagas' Disease

Our group continues to explore various aspects of the acute and chronic myocarditis associated with Chagas' disease. Two studies related to this disease were completed this year. The first of these showed that mast cells can be grown by culturing pieces of myocardium from mice chronically infected with T. cruzi, the causative agent of Chagas' disease, but not from the hearts of uninfected mice. Mast cells appeared in the medium during the first week of the culture in the area immediately surrounding the tissues and increased in numbers over time. The cells were identified as mast cells by electron microscopy and by staining with a variety of histologic and histochemical methods. Histopathological analysis of the cultured heart fragments from infected mice showed numerous mast cells, located mostly in areas where the histologic structure was abnormal and surrounded by fibrous connective tissue. This is the first report on the in situ proliferation and migration of mast cells from inflamed heart tissues. Mast cells grown from explanted cardiac tissue may be useful in developing in vitro models to study the role of these cells in various types of cardiomyopathies. The second study on Chagas' disease consisted of an analysis of the histologic and ultrastructural changes caused by infection with T. cruzi in myocardium and skeletal muscle of Calomys callosus (a small rodent). This animal model of Chagas' disease demonstrates two very interesting features: 1) macrophages appear to be the most important cellular element mediating the immune response, and 2) myocardial fibrosis develops very early in the time course of the infection but subsequently regresses to a considerable extent. The regression of myocardial fibrosis is a subject of great interest at the present time, particularly because of the potential role of collagenases (metalloproteinases) in mediating the lysis of interstitial collagens.

Cardiomyopathies

Cardiac AL Amyloidosis

Cardiac involvement by deposits of amyloid material constitutes an important cause of cardiomyopathy, particularly of restrictive cardiomyopathy. In this disorder, the amyloid material occurs in the form of fine fibrils that are deposited in the myocardial interstitium, the walls of the blood vessels, and even in the endocardium and the valves. A number of proteins can be involved in the pathogenesis of amyloidosis. However, various fragments of immunoglobulins are the most frequent and important causes of amyloid deposits (AL amyloidosis). This year a study was made, in collaboration with Dr. E. Arbustini and coworkers at the University of Pavia, Pavia, Italy, of the value of endomyocardial biopsy in the assessment of the severity and the clinical prognosis of cardiac AL amyloidosis. Histologic, immunohistochemical, ultrastructural and morphometric studies were made on myocardial biopsy specimens from 11 patients in whom the diagnosis of AL amyloidosis was based on the demonstration of a monoclonal immunoglobulinopathy and of amyloid deposits in tissues. Two types of myocardial deposits of amyloid material were recognized: perimyocytic deposits, localized along the basement membranes of the cardiac muscle cells, and interstitial nodular deposits. The latter were considered to be indicative of a greater severity of the disease process. Deposits associated with blood vessels did not seem to be of special prognostic value. This study demonstrated the usefulness of

endomyocardial biopsy in establishing the diagnosis of myocardial AL amyloidosis. Furthermore, myocardial biopsy also serves to characterize immunohistochemically the proteins in the amyloid fibrils and to asses the degree of myocyte damage and atrophy caused by compression of the myocytes by encircling deposits of amyloid.

Histiocytoid Cardiomyopathy

The clinical and morphological classification of cardiomyopathies in children, particularly in infants, continues to present difficult problems. Previous studies from our group have identified a distinctive type of cardiomyopathy that occurs in very small children and is characterized clinically by the development of sudden, often fatal arrhythmias and pathologically by multiple nodules of large, rounded histiocyte-like cells in epicardium and endocardium. We have previously shown that these cells are altered cardiac myocytes that contain few or no contractile elements but are filled with mitochondria. During the past year we have completed a study in which 3 new cases of this disorder are presented together with a review of the 50 other patients previously known to have this type of cardiomyopathy. Sixteen of the patients had well defined yellowish nodules on the endocardium, epicardium and/or valves. The other 37 had foci of abnormal myocytes throughout the myocardium. These cells were remarkably similar in all patients and had poorly developed or absent intercellular junctions, few or no contractile elements, and markedly increased numbers of mitochondria, which imparted a granular or vacuolated appearance to the cytoplasm. There was a high prevalence of anomalies involving the nervous system and eyes, and of oncocytic cells in various glands. This review presents evidence to exclude the possibilities that the histiocytoid cardiomyopathy represents a developmental anomaly of the atrioventricular conduction system, a multifocal tumor of Purkinje cells, a developmental arrest of cardiac myocytes and a diffuse type of mitochondrial cardiomyopathy. Histiocytoid cardiomyopathy is considered to be the result of hamartoma-like aggregations of cardiac myocytes with features similar to those of oncocytes. This syndrome is likely due to prenatal myocardial or systemic (viral?) Injury. Surgical excision of nodules of histiocytoid cells can result in clinical remission.

Apoptosis

During the past year we have undertaken several studies designed to evaluate, by means of electron microscopic and immunohistochemical techniques the occurrence of apoptosis in the cardiovascular system in normal and pathological conditions. Apoptosis (programmed cell death) is recognized morphologically by condensation of the nuclear chromatin, fragmentation of nuclear DNA, cell skrinkage and membrane blebbing. The DNA damage that leads to apoptosis can be recognized by the nick end labeling method, in which a terminal deoxynucleotidyl transferase is employed to add labeled deoxynucleotides to the terminal ends of strand breaks that characteristically develop in DNA in cells undergoing apoptosis. The initial applications of these techniques have been successful and have demonstrated labeling of apoptotic cells in the bulbus cordis of rats during normal embryonic development, in rats with doxorubicin-induced toxicity and in mice with pulmonary fibrosis caused by treatment with bleomycin.

Cardiac Differentiation

We have collaborated with Dr. R.E. Rodriquez and Dr. J.E. Parrillo of Rush-Presbyterian Medical Center in an ultrastructural and molecular study of the cardiac differentiation that is induced by triiodothyronine in the pluripotential P19 teratocarcinoma cell line. The pluripotential P19 teratocarcinoma cell line diffentiates into neurons when treated with 1μM retinoic acid (RA) and into cardiac muscle when treated with 1% dimethylsulfoxide or with low dose (1nM) RA. The effect of low dose RA prompted us to investigate the role of T_3 in the cardiac differentiation of P19 cells, since the thyroid hormone receptor belongs to the Steroid-Thyroid-Retinoic superfamily of transcription factors. Counts were made of the beating colonies that developed in culture of P19 cells, and a dose response curve showed that the optimal concentration of T_3 was 30 nM. These colonies were up to 3 mm in diameter, beat rhythmically for 4-6 weeks, and contained cardiac myocytes in which small nexuses were observed by transmission electron microscopy. Reverse transcription-polymerase chain reaction studies showed these cells to express the cardiac ventricular specific marker ventricular myosin light chain 2V. We also studied the role of P19 wild type transcription factors of the Steroid-Thyroid-Retinoic superfamily, which recognize the consensus sequence RGG(T/A)CA in DNA. These factors are also nuclear hormone receptors and discriminate among specific response elements in the DNA on the basis of the number of repeats of the consensus sequence, the distance between the repeats, and the 5'-3' orientation. Electrophoretic mobility shift assays showed a preference in T_3-treated cells for binding RGG(T/A)CA "half sites" in direct repeat orientation separated by 4 base pairs. The specificity of binding was confirmed in competition experiments. These findings suggest that thyroid hormone is capable of inducing P19 cells to differentiate, in a dose related manner, into spontaneously beating cardiac myocytes identified as such on the basis of ultrastructural criteria. The induction of differentiation is accompanied by an enrichment of nuclear receptors capable of recognizing RGG(T/A)CA direct repeats spaced by 4 base pairs in the DNA. This finding suggests that target genes bearing thyroid response elements spaced by 4 base pairs in their promoter regions play an important role in the cardiac differentiation induced by T_3 in P19 teratocarcinoma cells.

Valvular Heart Disease

Mitral Valve Allograft

The use of aortic and pulmonary valve allograft for the surgical replacement of diseased heart valves has become well established. However, the use of mitral valve allograft for the treatment of mitral valvular disease has continued to be the subject of considerable controversy, particularly with respect to their long-term durability. We have used the sheep model of valve implantation to make a detailed systematic evaluation of the modes of failure that occur in mitral valvular allograft implanted after treatment either with glutaraldehyde or with antibiotic-containing sterilizing solutions. Morphologic studies and calcium analyses were made on mitral valve allograft from 12 juvenile sheep surviving 12-24 weeks after mitral valve replacement. Before implantation, the allograft were treated with 0.625% glutaraldehyde (group 1, n=4) or with cold antibiotic solution (group II, n=8). Three group I animals died 12-19 weeks after implantation due to dysfunction of calcified valves; the surviving animal also had extensive allograft

calcification. One group II animal died with mitral regurgitation; the valves of the other 7 (including 5 with regurgitation) were explanted at 19-24 weeks. Chordal rupture related to calcific deposits was found in all group I valves. Leaflet perforations (n = 4) and ruptured chordae (n = 4), each due to connective tissue deterioration, were found in group II valves. Inflammatory reaction was absent or minimal in group I valves, but moderate or severe in group II valves. Fibrous sheaths were thicker in group II than in group I valves. Calcium levels were much higher in group I than in group II valves. This study showed that mitral valve allograft implanted in sheep have different modes of failure, depending on the nature of their preimplantation treatment. Allograft fixed with glutaraldehyde are likely to fail because of calcification and chordal rupture; those preserved with antibiotic-containing solutions, because of progressive deterioration of the structure of connective tissue.

Floppy Mitral Valves

The syndrome of idiopathic mitral valvular prolapse is characterized pathologically by floppiness of the mitral valve leaflets and often also of other components of the mitral valvular apparatus. Microscopically, this floppiness is manifested by various, poorly defined abnormalities in the valvular connective tissue. We have just completed a study aimed at defining the precise nature of the abnormalities that occur in mitral valvular connective tissue in patients with floppy mitral valves. Histological, immunohistochemical and ultrastructural studies were performed on 12 floppy mitral valves, 4 mitral valves showing focal myxomatous changes without prolapse, and 3 normal mitral valves. All floppy mitral valves were thickened by deposits of proteoglycans (which formed a network of stellate-shaped granules), but also showed diverse structural abnormalities in collagen (fragmentation of fibrils and presence of fibrils that were structurally abnormal and had a spiraling appearance) and elastic fibers (the association of microfibrils from elastin cores, granular appearance of the elastin and fiber fragmentation). This study showed that: 1) the structure of all major components of connective tissue in floppy mitral valves is abnormal; 2) alterations in collagen and accumulations of proteoglycans are nonspecific changes that may be secondary to the abnormal mechanical forces to which floppy mitral valves are subjected because of their excessively large surface area; 3) the presence of excessive amounts of proteoglycans may interfere with the normal assembly of collagen and elastic fibers, and 4) abnormalities of elastic fibers resemble those in other conditions characterized by structural dilatation or tissue expansion (i.e., emphysema).

Pulmonary Pathology

Several new studies were initiated in the past year in collaboration with Dr. Joel Moss and the staff of the Pulmonary and Critical Care Medicine Branch and Dr. William D. Travis, of the Department of Pulmonary Pathology of the Armed Forces Institute of Pathology. These studies concern various immunohistochemical and electron microscopic aspects of interstitial lung diseases, including idiopathic pulmonary fibrosis, pulmonary histiocytosis X, diffuse alveolar damage and pulmonary lymphangioleiomyomatosis. Two studies of experimentally induced pulmonary lesions (silicosis and bleomycin toxicity) were also completed this year.

Experimental Silicosis

This study was designed to characterize the nature, extent and time course of fibrotic and vascular alterations that develop in the lungs of rats with experimentally induced pulmonary silicosis. Ultrastructural and immunohistochemical studies were made of the lungs of rats given a single intratracheal injection of silica particles. Early lesions were characterized by accumulations of macrophages and neutrophils in alveolar lumina and interstitium, and damage to alveolar capillaries and epithelial cells. The initial lesions developed into granulomas and the damage to the alveolar lining was quickly repaired by proliferation of type II alveolar epithelial cells and bronciolar cuboidal cells. Vascular lesions consisted of endothelial damage and necrosis, and also began to be repaired quickly. The interstitium became massively fibrotic in many areas. Two patterns of endothelial cell migration were demonstrated. The first pattern was characterized by endothelial cells that extended their cytoplasm over preexisting, denuded basement membranes and replaced necrotic cells in alveolar capillaries. At 4 months after injury, some of these cells had developed fenestrations. The second pattern consisted of bud-like sproutings which developed only in peribronchiolar connective tissue. The observations in the present study indicate for the first time that peribronchiolar vessels are sources for renewal of alveolar capillary endothelium as well as for neovascularization.

Bleomycin-Induced Pulmonary Fibrosis

Bleomycin is an antineoplastic agent that is known to produce, in a dose-related manner, interstitial lung disease characterized by fibrosis and chronic inflammation. We have performed morphologic and morphometric studies to evaluate the protective effects of ICRF-187 against the pulmonary damage induced by bleomycin in male and female C57/BL6 mice (a strain that is known to be highly sensitive to the drug). Before administration of bleomycin, the animals received either saline or ICRF-187. The lungs of animals treated with bleomycin alone showed inflammation, hyperplasia of type II epithelial cells, squamous cell metaplasia and fibrosis. The extent of fibrosis was quantified by means of a color videometric system and histologic sections of lung stained according to a modified Masson trichrome method. The severity of these alterations, particularly of the fibrosis, was reduced in all groups of animals pretreated with ICRF-187. In vitro studies indicated that both ICRF-187 and its open-ring hydrolysis product (ADR-925) remove iron slowly from the bleomycin-iron complex that mediates the pulmonary toxicity of the drug.

ANNUAL REPORT OF THE CARDIOLOGY BRANCH
National Heart, Lung, and Blood Institute
October 1, 1994 through September 30, 1995

The experimental interests of the Cardiology Branch have continued to evolve over the past year as a result of an expanded emphasis of molecular-based research and of gene therapy, expansion of the recently added Section on Inherited Cardiac Diseases, and maintaintenance of our longstanding efforts in clinical and basic physiologic investigations.

MOLECULAR VASCULAR BIOLOGY

Increasing evidence implicates the smooth muscle cell (SMC) in the process of atherogenesis. Thus, it appears that SMCs are activated in response to injury, causing their proliferation and migration from the media to the intima. A similar but markedly accelerated process causes restenosis following coronary angioplasty in 25-50% of pts wks or months later. The focus of our research efforts is 1) to derive insight into the basic mechanisms responsible for the restenosis process and, 2) to develop novel molecular-based strategies to prevent restenosis.

Viruses and viral-mediated mechanisms as contributors to the development of restenosis and atheroscerosis: Last year we demonstrated that about one-third of restenotic lesions contained CMV DNA, and that an immediate early (IE) gene product of CMV, IE84, bound to and inactivated the tumor suppressor protein p53. This and other findings led us to hypothesize that CMV may predispose to restenosis, and that one of the mechanisms by which this could occur is by injury-induced reactivation of the latent virus leading to viral gene expression--subsequent interaction between IE84 and p53, as well as other CMV-mediated effects, could then increase the neointima, with resulting development of restenosis.

This year we futher explored the possible role of CMV in restenosis and in atherogensis. We first determined whether CMV increases the neointimal response to vascular injury in a rat carotid injury model. We found the neointimal thickness of CMV infected animals was nearly 40% greater than that of controls, a finding further suggesting that CMV plays a crucial role in the development of restenosis.

We next explored the mechanisms of the interaction between CMV and SMCs. Animal species have evolved specialized inflammatory cells that generate reactive oxygen intermediates (ROIs) to defend against pathogen invasion. We hypothesized that SMCs can also activate an ROI program as an antiviral cellular defense mechanism. SMCs were infected with CMV and intracellular ROI levels were assayed by a dye that fluoresces upon oxidation. CMV infection markedly increased ROI levels, an effect mediated in part by NADPH-oxidase (which is responsible for the neutrophile-mediated oxidative burst), the arachadonic acid cascade, and by xanthine oxidase. However, CMV has evolved counterstrategies to circumvent this SMC strategy to protect the organism from the lethal effects of the virus--thus, CMV actually *depends* on the SMC-induced ROIs for CMV gene expression, replication, and cytopathic effect, as evidenced by the suppressive effects of antioxidants on each of these processes. These findings suggest new strategies for inhibiting CMV's potential contribution to atherosclerosis and restenosis.

We also assessed the effects of CMV infection on 2 mechanisms playing a role in atherogenesis--SMC migration (SMC migration from the media to the neointima is a crucial event of atherogenesis and restenosis) and the accumulation of lipid-laden foam cells. We found that CMV infection, and specifically CMV's IE gene products: 1) increase SMC

migration, and 2) increase oxidized LDL uptake by SMCs, an effect in part caused by increased SR gene expression mediated by an IE72-induced transactivation of the SR promotor.

Because it not known how CMV is delivered to vessel walls and how it is activated once there, we also examined the following hypothesis: *CMV is transported by monocytes, which are recruited to sites of vascular injury, and the virus is activated by endothelial cell (EC) contact and by oxidized low-density lipoproteins (LDL).* We found that ECs and oxidized LDL, when in contact with monocyte precursor cells transfected with a reporter gene driven by the CMV major IE promoter, transactivate the CMV promoter. These results provide a link between CMV infection and the development of atherosclerosis, and also suggest another mechanism whereby oxidized LDL predisposes to atherosclerosis.

Gene therapy: We are continuing to explore the utility of adenoviral gene transfer to understand the mechanisms and to develop new treatment strategies for vascular diseases. Restenosis We previously we demonstrated that adenoviral gene transfer of the herpes simplex thymidine kinase (HS-tk) gene along with ganciclovir treatment can inhibit to the SMC response to vascular injury in the rat model of restenosis. We are now screening a variety of other adenoviral constructs including the bacterial gene cytosine deaminase and the tumor suppressor gene, p53, to assess their efficacy in preventing restenosis in the rat model, as well as the pig model of restenosis.

Angiogenesis See next section.

Signal transduction pathways: We have recently demonstrated that when SMCs are stimulated by PDGF they produce a burst of intracellular hydrogen peroxide. This rise in H_2O_2 can be attenuated by increasing the intracellular levels of catalase, the enzyme responsible for peroxide degradation, or by treatment with a chemical antioxidant (NAC). When the rise in H_2O_2 is inhibited, the ability of SMCs to respond to PDGF (tyrosine phosphorylation; mapk activity; SMC migration; DNA synthesis) is inhibited. These results suggest that H_2O_2 is a necessary intracellular second messenger for PDGF signal transduction.

We have recently been attempting to understand the pathway by which intracellular H_2O_2 is generated in cells. Preliminary evidence suggests that growth factor stimulation of H_2O_2 occurs through a ras and rac1 dependent pathway. This suggests that reactive oxygen intermediates (ROI) are downstream effectors of ras proteins. These results could therefor potentially explain a mechanism for the cardioprotective and cancer preventative effect of dietary antioxidants.

ENHANCEMENT OF CORONARY COLLATERAL DEVELOPMENT

Over the past 8 years we initiated studies designed to explore the possibility of enhancing collateral development to ischemic tissues caused by atherosclerotic occlusive disease. ***Angiogenic peptides***. We have shown that basic fibroblast growth factor (bFGF) and vascular endothelial growth factor, both angiogenic polypeptide growth factors, enhance coronary collateral development in dogs when administered repeatedly into the coronary circulation or, in the case of bFGF, when administered daily into the systemic arterial circulation for 7 days. These routes of administration, although acceptable in experimental animals, are not practical in human subjects. Most recently we showed that two single doses of bFGF administered into the left main coronary artery of dogs subjected to gradual occlusion of the left circumflex coronary artery enhanced coronary collateral flow by

39% ($P<0.0005$). This regimen would be feasible in human subjects. Based on these and a number of earlier investigations, we have obtained an IND (Investigational New Drug) for a Phase I study in human subjects. This is a randomized, double-blind safety study with a typical dose escalation format in which bFGF (or vehicle) is administered into the coronary circulation. The first pts entered into the study in July, 1995.

Gene therapy. We have begun to explore the possibility of using adenoviral gene transfer to deliver potent growth factors locally to areas of chronic ischemia to enhance collateral development. Gene therapy represents an attractive alternative to the administration of polypeptide growth factors, as sustained local expression of angiogenic gene products could result in site-specific angiogenic stimulation following a single intracoronary treatment. In our first study we demonstrated that adenoviral-mediated gene transfer of human VEGF to the ischemic hind limb of rabbits significantly improves collateral blood vessel function.

HYPERTROPHIC CARDIOMYOPATHY (HCM)

HCM is an inherited cardiac disease characterized by an increase in LV wall mass in the absence of another cause for hypertrophy. Because hypertrophy is a fundamental response of the heart and may be both adaptive and maladaptive, the study of HCM provides a window to an important physiologic cardiac process. Approximately 15% of cases result from a mutation in the beta myosin heavy chain (ßMHC), a dominant protein in the thick filaments of muscle fibers. These filaments interdigitate with thin filaments composed of actin and associated proteins. The two sets of proteins slide past each other to produce muscle contraction powered by the hydrolysis of ATP at the enzymatic portion of the myosin. In this actomyosin interaction a series of biochemical and corresponding structural changes occur in cyclic fashion. We have identified 30 mutations in the ßMHC gene and have described their distribution on the three-dimensional structure of myosin as solved by Rayment et al. Their clustering into 4 regions have suggested that they may interfere with the actomyosin crossbridge interaction in different portions of the cycle, thus leading to the inefficient molecular motors with different structural and functional defects. The physiologic study of these molecules casts light on the process by which chemical energy is transduced to directed movement in the actomyosin interaction. Other proteins in the contractile apparatus of muscle fibers cause HCM when mutated as well. In addition, the signaling pathway by which the inefficient structural and enzymatic portions of the contractile apparatus lead to hypertrophy is unknown; however, it is likely that these signaling events may also be inappropriately initiated and either modify or directly cause the hypertrophic process. Our laboratory is currently using the HCM families to pursue these lines of investigation.

Novel therapeutic strategies are also under investigation following the therapeutic success of DDD permanent pacing in adult pts with obstructive HCM. We have initiated a prospective study to examine the ability of DDD pacing to relieve LV outflow obstruction in HCM children between the ages of 5-15 years. To date, 38 children have been recruited and 1-yr follow-up in about half indicates that pacing is effective in improving both LV and RV obstruction and causes striking cardiac remodelling.

In addition, we have demonstrated that AV node ablation and implantation of rate responsive pacing systems improve symptoms and cardiac hemodynamics in about 40 HCM pts with paroxysmal and chronic AF, a serious complication in about 10% of HCM pts.

Finally, based on results of investigations into the role of angiotensin in hypertrophy, a prospective placebo-controlled randomized study is underway to investigate the ability of ACE inhibitor and angiotensin blockade in the Rx of HCM.

CONTRIBUTION OF THE ENDOTHELIUM TO VASCULAR TONE, PLATELET FUNCTION, AND MICROVASCULAR DYSFUNCTION

The endothelium secretes substances that dilate vascular smooth muscle and inhibit platelet aggregation. The Section on Cardiac Catheterization and the Echo and Clinical Physiology Lab have continued their studies designed to elucidate 1) the regulation of microvascular function in the coronary and systemic circulation of humans, 2) the mechanisms by which the endothelium normally modulates coronary and systemic vascular tone and influences platelet function, and 3) how its dysfunction contributes to the development of various disease states.

Endothelial Function in Hypertension and Hypercholesterolemia: We previously demonstrated that pts with essential hypertension and pts with hypercholesterolemia have a reduced vasodilator response to acetylcholine (ACH), an endothelium-dependent dilator, compared to normal controls. This abnormality is related to reduced bioactivity of nitric oxide (NO). It has been proposed that a potential mechanism for impaired endothelial regulation of vascular tone in certain cardiovascular conditions is an increased breakdown of NO by superoxide anions. We therefore hypothesized that a similar mechanism might operate in hypertensive and hypercholesterolemic pts. To this end, we investigated the effect of superoxide dismutase (a scavenger of superoxide anions) on endothelium-dependent vasodilation. We used native copper-zinc superoxide dismutase (CuZn SOD) which, given its negative charge, has poor or no intracellular penetrance, thus allowing the study of extracellular breakdown of NO by superoxide anions. Our studies showed that the impaired endothelium-dependent vasodilator response of hypertensive or hypercholesterolemic pts is not modified by CuZn SOD, thus indicating that extracellular destruction of NO does not play a significant role in the impaired endothelial vasodilator function in these conditions.

An important source of superoxide anions in the intracellular compartment is the xanthine oxidase system. We therefore determined whether increased production of superoxide anions from this source might contribute to the blunted endothelium-dependent vasodilation of hypertensive and hypercholesterolemic pts. Oxypurinol (an irreversible inhibitor of xanthine oxidase) increased the response to ACH in hypercholesterolemic pts, without effecting the response to sodium nitroprusside (an endothelium-independent agent). These results support the concept that xanthine oxidase-generated superoxide anions might contribute to the endothelial dysfunction of pts with hypercholesterolemia. In contrast, we observed no significant modification of endothelium-dependent responses by oxypurinol in hypertensive pts.

Regulation of microvascular function in the coronary and systemic circulation of humans. In previous years we primarily investigated pts with angina-like chest pain and potential ischemic syndromes in the absence of epicardial coronary artery disease (CAD), demonstrating that a subset of this pt population has coronary microvascular dysfunction. Over the past 3 years, we have focused our efforts on estrogen-deficient, postmenopausal women. We demonstrated that estrogen importantly affects endothelium-dependent vasomotion of both coronary and systemic (forearm) circulations of estrogen-deficient postmenopausal women when acutely administered. Estrogen also protects low density lipoprotein from oxidation when administered acutely and chronically to postmenopausal women. We are currently investigating the antioxidant effects of several estrogen preparations. Because progestin compounds may counteract many of estrogen's biological effects, we are studying the impact of progestins on estrogen's antioxidant effects.

Preliminary data from our laboratory suggests that the vascular effects of estrogen administered at physiologic levels are mediated through potentiation of the production or release of NO. A study is in progress to further clarify this mechanism. In the same study we will investigate whether estrogen's antioxidant effects are mediated through the release of NO. These studies will be performed both in the forearm and in the coronary circulations.

As we continue to perform studies of coronary microvascular dynamics using endothelium-dependent and endothelium-independent agonists at the time of cardiac catheterization, we are attempting to demonstrate whether or not such studies identify pts with objective evidence of myocardial ischemia during stress using transesophageal echocardiography and dobutamine stress. Because most pts with chest pain and normal coronary angiograms are women, we will determine whether dobutamine stress testing has greater sensitivity and specificity than other conventional noninvasive testing in identifying women likely to have normal coronary angiograms at cardiac catheterization.

We are also interested in mechanisms of pain in pts with chest pain and normal coronary angiograms, based on our observation that as a group, these pts commonly have exaggerated or abnormal cardiac pain sensitivity. In collaboration with Dr. John Stuhlmuller, we are performing studies of pain pathway activation in the central nervous system using oxygen-15 labeled water and positron emission tomographic imaging both at rest and during dobutamine stress. We are also investigating the role of endogenous opioids in mediating activation of pathways in pts with chest pain and normal coronary angiograms compared to normal volunteers and pts with CAD with symptomatic angina and silent ischemia.

Relation between CAD risk factors and endothelial dysfunction: We had previously demonstrated depressed NO activity in pts with risk factors for atherosclerosis and those with established atherosclerosis. The present studies demonstrate that the abnormal response of the coronary and peripheral vasculature to ACH in this pt subset is not restricted to the muscarinic receptor and extends to other receptors, such as that for substance P. We also examined NO activity in human saphenous vein grafts in vivo. Basal and stimulated NO activity was reduced in the grafts compared to coronary arteries. The responsiveness of the grafts to the exogenous NO donor, sodium nitroprusside, was also depressed. Thus, despite arterialization of veins after their use as vein grafts during bypass surgery, NO activity remains reduced compared to arterial conduits.

We also investigated the importance and mechanisms of platelet activation in the morning hours to the increased incidence of cardiovascular events at this time. Platelet activation occurred during arousal and assumption of upright posture in the morning hours using whole blood platelet aggregation. However, this was not accompanied by activation-dependent platelet surface receptor changes measured by flow cytometry. Thus, the increases in platelet count, fibrinogen levels and catecholamine levels that occur in the morning hours, and the reducted Hct is responsible for promoting platelet aggregation during standing. To examine the role of catecholamines in producing increased platelet activation upon arising and during exercise, the effect of Yohimbine, an alpha-2 receptor antagonist, was studied. Yohimbine inhibited the increase in platelet activation with upright posture but not with exercise. These studies help define the mechanisms of increased platelet aggregation that occurs during assumption of upright posture and with exercise, and may define new therapeutic approaches in reducing the morning incidence of acute coronary and vascular events.

We have also studied the prognostic significance of myocardial ischemia that occurs during normal daily activities and during treadmill exercise testing in a low risk subset of pts with coronary artery disease. Four-year follow-up was performed in 214 pts who had been

treated medically because they were deemed to be at low risk on medical therapy. Neither the presence of ischemia during Holter monitoring nor during exercise correctly predicted increased risk of future cardiac events.

CORONARY ARTERY DISEASE AND HEART FAILURE

In pts with chronic CAD and congestive heart failure, the distinction of ischemic but viable from nonviable myocardium is an issue of increasing clinical relevance in guiding medical or interventional therapy.

Biochemical Detection of Viable Myocardium: An increase in ^{18}F-deoxyglucose (FDG) uptake in asynergic regions with reduced blood flow provides a metabolic signal for viable myocardium, capable of improving after revascularization. Whether the magnitude of reduction in regional blood flow assessed by ^{15}O-water positron emission tomography (PET) can also discriminate viable from nonviable myocardium has not been established. Among pts with chronic CAD undergoing revascularization, we performed pre-operative PET at rest with FDG and ^{15}O-water and pre- and post-operative gated magnetic resonance imaging (MRI) and radionuclide angiography. Mean LV ejection fraction increased from 30 $\pm$ 13% pre- to 35 $\pm$ 11% post-revascularization. In regions with abnormal systolic wall thickening preoperatively, mean regional blood flow was similar in regions that improved compared to regions that did not improve after revascularization. However, mean FDG uptake was significantly higher in regions that improved compared to regions that did not improve after revascularization. Our findings suggest that reliance on the magnitude of blood flow as assessed by ^{15}O-water (expressed per gram of perfusable myocardium) is insufficient for predicting recovery of function after revascularization. On the other hand, metabolic assessment with FDG in asynergic regions provides unique insights into myocardial viability.

Congestive Heart Failure: Myocardial fibrosis is found in pts with endstage cardiomyopathy (CM). It is possible that in the natural history of congestive CM, progression to fibrosis is preceded by a period of functional reversibility. Hibernating myocardium seen in pts with ischemic CM may represent such a period of functional recovery. We found that among symptomatic pts with heart failure and LV dysfunction, there was a significant difference in regional blood flow and metabolism among pts who had ischemic CM compared to those with nonischemic CM. In the ischemic CM pts, 57% of all regions demonstrated abnormal blood flow and almost 50% of such regions exhibited preserved glucose extraction indicative of ischemic and viable myocardium. In contrast, among the nonischemic CM pts, decreased regional blood flow occurred in approximately one-fourth of regions and the majority (89%) of such regions had matched decrease in glucose extraction (nonischemic). Thus, despite the absence of significant epicardial coronary artery stenosis, decreased regional blood flow occurs in pts with nonischemic CM and may reflect regions with admixture of viable myocytes and fibrosis.

Histomorphologic studies of explanted hearts: Among pts with severe ischemic CM waiting for cardiac transplantation (CT), we correlated total volume fraction of collagen in the mid LV with indices of LV systolic and diastolic function derived from exercise radionuclide angiography studies performed before CT. Following CT, pathologic slices for each pt were weighed and the distribution of fibrosis from midventricular slices were studied quantitatively after staining with Picro Sirius red. Mean thickness of the LV wall correlated inversely with the volume fraction of collagen in the non-infarct region of the LV. Our data also suggested that the non-myocyte compartment of the non-infarct region of the LV myocardium undergoes significant remodeling in chronic ischemic CM which may contribute to the changes in LV systolic and diastolic dimensions and function.

Transesophageal dobutamine stress echocardiography:

CAD: We have been investigating the quantitative myocardial contractile response to increasing doses of dobutamine to determine the clinical usefulness of dobutamine stress echocardiography. In pts with CAD a critical reduction in blood flow has been proposed as the mechanism responsible for *chronic* LV dysfunction. To test this hypothesis, we have investigated the relation between the effect of coronary stenoses on resting systolic function and on myocardial contractile reserve during adrenergic stimulation. If a reduction in blood flow accounted for the diminished myocardial performance, then the magnitude of systolic dysfunction at rest would be related to the inotropic response to dobutamine. Instead, we demonstrated no correlation between basal and stimulated myocardial contraction, suggesting that limitation of blood flow is unlikely to be the sole mechanism responsible for chronic LV dysfunction in CAD.

We previously reported that dobutamine frequently provokes both the characteristic chest pain experienced by CAD pts during their daily life and the ECG abnormalities observed during exercise. However, no regional wall motion abnormalities were observed in these pts. To more precisely assess whether these pts have mild degree of ischemia as the cause of their symptoms, we analyzed the quantitative myocardial contractile response to dobutamine. No difference was observed between pts with chest pain and normal coronary arteriograms and the normal controls. These findings strongly suggest that myocardial ischemia is not responsible for the chest pain symptoms of these pts and support the concept of abnormal cardiac nociception in this condition.

Aortic regurgitation (AR): In pts with chronic severe AR and normal LV function, valve replacement is not recommended until symptoms, LV dysfunction, or progressive LV dilatation develop. However, whether concealed myocardial dysfunction can occur before those more obvious indices of deterioration are detected has not been established. We therefore studied the myocardial contractile response to dobutamine with transesophageal echocardiography in such pts. Compared to normal controls, pts with AR have markedly reduced inotropic reserve, which is not related to the magnitude of afterload changes and may represent an early stage of myocardial dysfunction.

ANNUAL REPORT OF THE HEMATOLOGY BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1994, TO SEPTEMBER 30, 1995

The major research and clinical interests of the Hematology Branch are in normal hematopoiesis d the pathogenesis, pathophysiology, and effective treatment of bone marrow failure and malignant ood cell diseases. The Branch consists of research groups in the following areas: B19 parvovirus; ne marrow failure states, including acquired and constitutional aplastic anemia and involving viral and mune studies; gene therapy, including retroviruses and adeno-associated virus vectors; and bone rrow transplantation. Included in the Hematology Branch is the 2 West clinical service and the Bone rrow Transplant Unit. The major diseases studied include acquired aplastic anemia, Fanconi's emia, chronic myelogenous leukemia, multiple myeloma, acute myelogenous leukemia, elodysplasia, and congenital hemoglobinopathies. The Hematology Branch also administers an redited and successful hematology fellowship program.

9 PARVOVIRUS

B19 parvovirus is a human pathogen that is responsible for a number of diseases, including fifth ease in children, transient aplastic crisis in patients with sickle cell anemia and other underlying molytic states, a rheumatologic syndrome in adults, and pure red cell aplasia in immunosuppressed lividuals. We previously identified the target cell of B19 parvovirus as the human erythroid ogenitor cell. The virus' extraordinary tropism for this cell is largely mediated by its receptor, throcyte P antigen. A defective humoral immune response is responsible for persistent infection.

inical Studies

In collaboration with our industrial partner, MedImmune, phase I trials of a recombinant vaccine gent produced in a baculovirus system were initiated in the spring of 1995. Normal volunteers have ceived increasing doses of empty parvovirus capsids enriched for the highly immunogenic minor uctural protein, to date without toxicity. Antibodies as determined by ELISA were formed at all dose vels and neutralization studies are underway. The identification of simian parvovirus and our servation that B19 and simian parvovirus can cross-infect cells from other species have lead to the velopment of an animal model which may be useful in both vaccine development and in the analysis the effects of parvovirus infection on the fetus.

ther Animal Parvoviruses

Two monkey parvoviruses have been discovered, simian parvovirus in cynomolgus monkeys and different but related virus termed pigtail macaque parvovirus. Sequencing of simian parvovirus has vealed marked homology with the human virus, and similar studies are underway for the macaque rus. In addition, we have cloned and sequenced goose parvovirus and discovered an unexpected mology to adeno-associated virus, despite the fact that goose virus is clearly autonomous and not pended on a helper virus for replication. Production of chimeric goose-AAV viruses as gene vectors s been undertaken.

ructural and Molecular Studies

Efforts to determine the fine crystal structure of B19 parvovirus are continuing with laboration with colleagues at Purdue University. Cyro-electromicrographs have revealed that B19

ovirus binds globoside, probably at the 5-fold axis of symmetry in a large cavity formed in the ence of the usual parvovirus spike. Studies of the nonstructural protein of B19 parvovirus have gressed using the two-hybrid yeast system, which has shown interaction between this protein and -3, an important transcriptional regulator.

leno-Associated Virus

Only P antigen as the B19 parvovirus cellular receptor has been identified as a binding site for Parvoviridae. Studies of adeno-associated virus using both overlay and suspension assays have own specific binding to a 150 kilodalton cell surface protein; enzymatic studies have suggested rbohydrate may be the binding site. Surprisingly, human CD34 cells have few if any binding sites, t otherwise there is a good correlation between the presence of the 150 Kd protein and permissivity infection studies. AAV-3 does not appear to use the same receptor.

ONE MARROW FAILURE

Aplastic anemia has clinical and laboratory features to suggest that viruses or drugs may incite a thophysiologic immune response. We have previously demonstrated the presence of activated totoxic lymphocytes that over-express τ-interferon in the blood and bone marrow of patients; these lls and lymphokines are the targets of successful immunosuppressive therapy. In Fanconi's anemia, contrast, aplastic anemia occurs as a result of genetic lesion, probably in a protein required for DNA pair. This patient population is amenable to gene therapy.

olecular Mechanisms for Immune Suppression of Hematopoiesis

Both gamma interferon and tumor necrosis factor-α are over-expressed in the bone marrow of ients with aplastic anemia. Using in vitro methods, we have investigated the downstream events ssibly involved in hematopoietic suppression by T cells. We showed that the Fas receptor is pressed on hematopoietic cells. Fas is a cell surface antigen first identified in the immune system as ggering apoptosis on binding of its ligand or a specific monoclonal antibody. For hematopoietic lls, Fas is induced in total bone marrow by inhibitory cytokines, analogous to Fas expression with tivation of lymphocytes. On primitive cells as defined by the CD34+ antigen, Fas expression is rmally minimal but greatly stimulated by addition of gamma-interferon or tumor necrosis factor- ha. Monoclonal antibody to Fas is synergistic with these two negative regulators of hematopoiesis in ppressing hematopoietic colony formation and at the level of the stem cell, as monitored by the long- m cultured initiating cell assay. In the CD34+ population, Fas expression and triggering is sociated with apoptosis. In addition to the Fas system, we have also implicated nitric oxide as a tent downstream inhibitor of hematopoiesis: CD34+ cells contain nitric oxide synthase and are sceptible to nitric oxide inhibition.

Three important aspects of interferon action on hematopoietic cells have been expeimentally monstrated. First, we have shown that both interferon and tumor necrosis factor are inhibitory of matopoietic cell proliferation at all stages of differentiation, from mature progenitors to stem cells, as termined in long-term culture initiating surogate assay. Second, we have shown that these inhibitory fects are secondary to induction of apoptosis through the Fas ligand pathway; both induction of nitric ide synthase and the interferon regulatory factor-1 have been identified as functioning downstream gnalling pathways. Third, the local effects of α-interferon on hematopoiesis have been studied in g-term bone marrow culture, in which stromal cells have been genetically engineered to constitually press low levels of τ-interferon. Under these circumstances, abrogation of stem cell generation in the

adherence cell layer occurs at 100-fold less concentrations of endogenous interferon compared to genous cytokine. As expected both Fas induction and apoptosis occur in CD34 cells in the nadherent cell layer.

Putative Virus in Hepatitis/Aplasia

We have studied the relationship of a novel hepatitis virus termed GBV-C in collaboration with t discover at Abbott Laboratories. GBV-C is a member of the pesti- or flavivirus family. We have rd reverse gene amplification to detect and sequence this virus in specimens from patients with plastic anemia, hepatitis/aplasia, and fulminant hepatitis. Preliminary data indicate that the virus is prevalent in all populations and probably etiologic in some of these syndromes. In addition, virus prevalent studies have been conducted in sera from Vietnam and suggest a rate of approximately 4% in h normal population. Sequence studies have suggested a high degree of geneotypic variability among GBV-C strains, and in addition, our current reverse PCR technique almost certainly does not detect all virus genotypes. Clinical observations have suggested that both the fulminant hepatitis and hpatitis/aplasia syndrome are immunologically mediated, as these patients respond to immunosuppressive therapy.

Epidemiology of Aplastic Anemia

An extensive NHLBI epidemiologic study of aplastic anemia in Bangkok and to rural regions has been completed. Important associations established include relationships to low socioeconomic status, gain farming, and to solvent and some drug exposures. Serum samples from both Thailand and Vietnam are available for serologic testing.

Treatment of Acquired Aplastic Anemia

Our immunosuppressive trial of intensive treatment with ATG and cyclosporin has now enrolled over 70 patients. There is an apparent plateau in survival, although adverse events, mainly paroxysmal nocturnal hemoglobinuria, continue to occur years after hematologic responses. Correlations with stem cell number, as determined by the LTCIC assay, are continuing. In studies of growth factors, we have observed one clinical significant improvement in a patient with refractory aplastic anemia treated with stem cell factor, a novel cytokine that affects primitive hematopoietic stem cells.

Paroxysmal Nocturnal Hemoglobinuria

PNH is associated with aplastic anemia. In this disease, there is absent presentation of glycophosonositol proteins to the cell surface due to deletion mutations in X-chromosome gene called PIG-A. In collaboration with colleagues at Case Western Research Center, we have created a "nockout" embryonic stem cell in which the murine equivalent of this gene is non-functioning. These cells fail to form embryonic bodies and to differentiate into hematopoietic cells in vitro. However, this defect can be corrected by co-culture with normal embryonic cells. Under these circumstances hematopoietic cell differentiation can be achieved from the knockout clone. These results imply that embryogenesis but not hematopoiesis is abnormal in the absence of functional PIG-A gene product. One interesting hypothesis is that the required cell surface proteins are transferred from normal to knockout cells.

Fanconi's Anemia

The identification of the Fanconi's anemia type C gene has allowed in vitro and in vivo studies of the genetic defect in this form of constitutional aplastic anemia. In cell culture experiments, we have

monstrated that the FACC gene can be transiently and permanently transduced into hematopoietic cell
s and primary CD34+ cells from patients with this type of Fanconi's anemia and correct the
enotype of the disease, including the cytogenetic abnormality and the proliferative deficient. Over-
pression of the Fanconi's C gene in transgenic mice had no adverse effects on development, may
nfer relative resistance to mitocycin D.

The biologic activity of the Fanconi's C gene product is unknown. We have succeeded in
pressing the protein in a baculovirus system, and yeast two hybrid experiments are underway. The
nconi's C gene has been demonstrated to correct the susceptibility of Fanconi's anemia patient cells
SV40 viral transformation. Interaction with proteins recognized as important in the transformation
chanism is underway.

Finally, we have instituted a gene therapy protocol for Fanconi's anemia type C patients. These
tients undergo treatment with the growth factor granulocyte colony stimulating factor followed by
tokine and repeated ex-vivo transduction with a retrovirus vector containing the Fanconi's C gene.
e first patient has received the initial round of therapy without evidence of clinical toxicity. Analysis
transduced CD34+ cells have shown that approximately 50% of progenitors were marked prior to
usion.

ENE TRANSFER

ene Transfer in Primates

Our large non-human primate facility at 5 Research Court performs approximately two gene
sfer experiments each month in irradiated, myeloablated animals. In general, problems with
oable radiation-induced vasculitis have resolved and mortality due to the bone marrow transplant
ocedure is now low. Experiments have been directed toward improving gene transduction by
ection of target cells and testing of various vectors prior to their employment in humans. For these
rposes, a number of novel technical advances have been made, including development of a method
luekopherisis of small animals and a technique for simultaneous DNA analysis and multiple
rimetric immunophenotyping.

More detailed fractionation of the CD34+ hematopoietic cell population has not proven to be an
provement over the use of the CD34+ cells prepared by immuno selection with the CellPro immuno
sporbtion system. Also used as cell targets have been CD4-enriched lymphocytes obtained by
kopherisis. Transduction procedures for both CD34+ and CD4+ cells have been optimized.

Several novel vectors have been tested. Adeno-associated virus has been used for gene
nsduction for the first time in primates, but todate the transduction efficiency appears low, and
nparable to that obtained with retroviral vectors. Relatively high transduction efficiencies have been
ained using a recombinant retroviral vector based on Gibbon ape leukemia virus. In several animals,
narkedly high transduction rates have been achieved, with approximately 10% of circulating
kocytes positive at day 100.

ne Transfer into Humans

Our Branch has conducted a major test of gene transduction into human hematopoietic progenitor id stem cells in patients. A total of 17 patients undergoing autologous stem cell transplantation as eatment for breast cancer or multipe myeloma have been studied. Blood and bone marrow cells were arked with myosin-resistance genes and the presence of these genes in the circulation and marrow ve been assesssed periodically after transplant. These studies have the purpose of assesssing the lative efficiency of gene transduction into blood and bone marrow, levels of gene expression, and the urce of relapse in patients with malignant disease after autologous transplantation. To date, 14 of 17 tients have shown evidence of hematopoietic cells containing the transferred gene, and 5 patients have own the presence of the marker for one year after the procedure. However, the level of marked cells extremely low. In collaboration with investigators in the National Institute of Neurologic Disease and roke, we have initiated a therapeutic Gaucher's disease gene therapy protocol for introduction of the ucose glucocerebrosidase gene into CD34+ cells.

The relatively inefficient gene transduction by retroviral vectors is a major problem in gene erapy. In the laboratory, we have studied methods to improve gene transduction in vitro. We monstrated that gene transduction can occur in a serum-free environment with highly purified vector eparations, which is of importance in reducing the lengthy process of regulatory approval. One novel proach has been to block cytokine inhibition of primitive stem cell cycling, as retroviral vectors quire mitosis for integration.

NE MARROW TRANSPLANTATION

Our Bone Marrow Transplant Unit has clinical and research arms. We have conducted a major inical trial of delayed add-back of donor lymphocytes in an effort to prevent acute graft-versus-host sease yet retain therapeutically beneficial graft-versus-leukemia effects. In this study, patients with ute and chronic myelogenous leukemia or myelodysplastic syndromes received bone marrow depleted mphocytes from fully histocompatible siblings; donor lymphocytes are infused at days 30 and 45. A tal of 30 patients have now been transplanted. Only two relapses have occurred, both in a high-risk oup, suggesting that the protocol retains graft-versus-leukemia efficacy. Acute graft-versus-host sease has only occurred in two patients. In contrast, in four patients in whom T cell depletion was t feasible, all developed acute graft-versus-host disease. Over 60% of patients have survived free of sease to date. However, a major problem has been the occurrence of cytomegalovirus infection, and eumonia due to this virus was a cause of death in several cases. This procedure appears to be safe d effective, and viral infection may be preventable by prophalytic medical therapy.

In the laboratory, efforts are directed at functional depletion of those lymphocytes responsible r graft-versus-host disease with retention of graft-versus-leukemia cells. The most promising result s been that both proliferative responses and helper lymphocyte precursor cells that recognize the cipient are selectively depleted after antibody treated using a toxin-congrugated antibody that is ecific for activated T cells. In related experiments, efforts have been directed at characterizing tigens present on leukemia cells that would serve as targets for the T cell response. We examined ptide sequences that were predicted to bind to HLA molecules to specific HLA molecules. Synthetic tides have been tested in binding assays of class I or class II defective cells. Strongly binding ptides have been used to generate peptide-specific T cell clones in vitro. Several proteins have

ded peptides that bind avidly to HLA molecules and induce T cell proliferative responses. The tide representing the BCR-ABL fusion protein, which is unique to chronic myelogenous leukemia ls, has been used to generate T cell clones which are cytoxic to target cells expressing this leukemia cific peptides.

ANNUAL REPORT OF THE HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October I, I994 through September 30, I995

The Hypertension-Endocrine Branch studies the mechanisms of blood pressure regulation and the causes and treatment of hypertension and its complications. In the last year, the second of our two Israeli scientists returned home, and we added a tenure track scientist in order to bring the tools of molecular biology to bear on our research efforts. Our efforts have been concentrated in three areas: I) vasoactive substances, mainly endothelin, 2) pheochromocytoma, and 3) molecular studies of the basis for the relationship between hypertension, insulin resistance, and obesity.

I. Vasoactive substances.

A. Renin angiotensin system. Congestive heart failure (CHF) is a frequent complication of hypertension. It is a pathologic condition, characterized by avid sodium retention and edema formation. The exact mechanisms underlying the exaggerated sodium reabsorption in CHF have not been clarified. Several mechanisms have been implicated, including the renin-angiotensin system (RAS). Therefore, we studied the expression of various components of the local RAS by quantitation of cardiac, renal and pulmonary mRNA levels of renin, angiotensin converting enzyme (ACE) and angiotensin II receptor subtypes (Types AT-1 and AT-2) in rats with different severities of CHF, induced by the creation of an aortocaval fistula (ACF). After creation of an ACF, rats either compensate and maintain normal sodium balance, or decompensate and develop sodium retention with extensive edema formation and progression to death. The importance of the RAS system in CHF is demonstrated by the fact that decompensated animals can be saved and converted to compensated CHF by treatment with either ACE inhibitors or renin inhibitors. Both groups developed cardiac hypertrophy, which was greater in decompensated rats. Reverse transcription, followed by quantitative polymerase chain reaction (RT-PCR), revealed that renal renin-mRNA increased 52% in compensated animals and I30% in decompensated rats. Renin mRNA levels in the myocardium also increased 68% and I40% in rats with compensated and decompensated CHF, respectively. ACE-mRNA levels increased in a similar pattern in the heart, but not in the kidneys. Moreover, renal and cardiac ACE immunoreactivity levels, assessed by western blot analysis, showed the same trend. AT-1 receptor mRNA levels decreased 54% only in the myocardium of decompensated rats, while AT-2 receptor mRNA did not change in any tissue studied. These studies clearly show that CHF is associated with remarkable increases in the expression of the local RAS, especially in the heart. This may well explain the success of ACE inhibitor therapy in preventing myocardial remodeling and the progression of heart failure in human subjects with either hypertension and/or congestive heart failure.

B. Endothelin. Endothelin (ET) is an extremely powerful vasoconstrictive peptide, produced by vascular endothelium. Cyclosporin A (CsA) is an immunosuppressive agent widely used to prevent allograft rejection. However, it has several serious side effects, which include acute and chronic nephrotoxicity and

hypertension. Early studies have suggested that endothelin may be the cause of CsA-induced nephrotoxicity and hypertension, but an interaction between the two has never been proven. Rats given CsA for six days developed severe renal insufficiency, associated with a fall in glomerular filtration rate of 74%. Urinary excretion of ET increased from an undetectable level to 3l.7 pg/day, while plasma levels of ET were unchanged. Neither acute nor chronic treatment with CsA affected either the clearance of ^{125}I-ET-1 from the blood or the renal and pulmonary uptake of the peptide. Moreover, CsA did not affect the degradation of radiolabelled ET-1 by highly purified recombinant neutral endopeptidase, a well-known endothelinase. In addition, reverse transcription, followed by quantitative polymerase chain reaction (RT-PCR), revealed that ET-1- mRNA in the renal medulla increased 59%, while the expression of both ET-3 and ECE was unchanged. In earlier studies, we had shown that radioactive ET is not cleared into the urine except as fragments, showing that all urinary ET is produced in the kidney. Our current findings show that the elevated urinary ET levels after CsA treatment originate from the kidney and reflect increased renal synthesis of ET. In addition, they show that the production of ET is regulated at the level of mRNA transcription and the expressions of ET-1 and ET-3 are regulated independently. Therefore, it would seem likely that the increased ET production in the kidney, in association with CsA administration, is a reflection of the nephrotoxicity and not its cause.

C. Mechanism of action of endothelin. Endothelin is an extremely powerful vasoconstrictor peptide that induces characteristically long-lasting contractions. We used both intact and endothelium-denuded rat aortic rings to investigate the role of protein kinase C (PKC) in ET-induced contractions. Very low doses (10^{-9}) of ET produced a gradual and sustained contraction of greater magnitude in denuded aortic rings than in intact rings. These ET-induced contractions were inhibited by three different PKC inhibitors, and inhibition was always greater in intact than in endothelium-denuded aortic rings. Pretreatment of aortic rings with a PKC activator potentiated ET-induced contractions. These results strongly suggest that PKC mediates ET-induced contractions in rat aortic rings and that an intact endothelium is required for maximum inhibition by PKC inhibitors.

ET, like many other powerful vasoconstrictors, causes the release of modulating vasodilators including prostaglandins, especially prostacyclin. The mechanism for this vasodilator release remains unclear. Very small doses of ET, administered to rat aortic rings, produced a ten-fold increase in prostacyclin release. This prostacyclin release was inhibited completely by the same three PKC inhibitors that blocked ET-induced contractions. As expected, ET-induced prostacyclin release was also blocked by pretreatment with inhibitors of either phospholipase A_2 or cyclooxygenase. Both enzymes are involved in the production of prostacyclin precursors. These results raise some fascinating questions: l) What is the common link between the ET-induced contraction and prostacyclin release? 2) Why is an intact endothelium required for maximal inhibition of ET-induced contractions by PKC inhibitors? 3) Is an increase in intracellular calcium the connection between ET-induced vasoconstriction and PGI_2

release? We shall attempt to answer these questions.

II. Pheochromocytoma. We have continued our efforts to improve the diagnosis, localization, and treatment of pheochromocytoma. In collaboration with the Clinical Neuroscience Branch, NINDS, we have evaluated the clinical usefulness of a new technique for measuring free metanephrines, i.e., metanephrine (MN) and normetanephrine (NMN) in blood by means of liquid chromatography and electrochemical detection. We have applied this new technique to patients with pheochromocytoma, patients with both essential and other forms of hypertension, and to normotensive control subjects. We compared the results of plasma MN with measurements of plasma catecholamines and urinary MN. We showed that a pheochromocytoma results in a considerably larger increase in plasma MN than in plasma catecholamines. Plasma NMN concentrations were increased in all patients with pheochromocytoma, whereas MN concentrations were increased in only some of the patients. No patient with a pheochromocytoma had plasma concentrations of both NMN and MN that were within the normal range. Thus, plasma MN never yielded false negative results, i.e., 100% sensitivity. For equal sensitivity, plasma MN provided significantly better specificity than did either plasma catecholamines or urinary MN. The finding of normal plasma MN excluded the diagnosis of pheochromocytoma, whereas the finding of normal plasma catecholamines or normal urinary MN did not. Therefore, measurement of plasma MN provides the most sensitive, single clinical test yet described for the diagnosis of pheochromocytoma. We propose to continue the evaluation of the usefulness of this new assay in the diagnosis of pheochromocytoma.

In collaboration with the Urologic Oncology Section, Surgery Branch, NCI, we have studied the clinical characteristics and management of pheochromocytoma associated with von Hippel-Lindau (VHL) disease. Pheochromocytoma in VHL kindred are associated with specific missense mutations in the VHL gene. Kindred with these specific mutations are thus prone to develop pheochromocytoma. We screened VHL kindred to detect early pheochromocytoma, and this allowed us to clinically characterize these patients and their pathology. One hundred and twenty-two individuals with VHL disease were evaluated at NIH. Thirty patients had a history of either previously resected pheochromocytoma or new findings consistent with this diagnosis. Sixty-three percent of VHL patients with pheochromocytoma had multifocal tumors and 26% were from a single VHL kindred. Only seven of nineteen patients with newly diagnosed adrenal masses had signs or symptoms typical of pheochromocytoma. The remaining twelve patients were judged likely to have pheochromocytoma based on characteristic radiographic findings. Urine catecholamine values and provocative/suppression physiological testing confirmed the diagnosis in six of these patients, each of whom have had their pheochromocytoma resected. Seven patients with normal or equivocal biochemical studies are being managed expectantly with interval imaging and laboratory studies. Some patients were observed to have tests change from negative to positive over time, as adrenal masses enlarged on CT. From these studies, we conclude that pheochromocytoma, associated with VHL and detected through screening of affected kindred, are frequently asymptomatic or nonfunctional. Small, early VHL

pheochromocytoma appear to be biochemically inactive by standard testing and become more functional with time. VHL kindred, particularly those with a history of pheochromocytoma, must be screened frequently and repeatedly in order to prevent potential morbidity and mortality from an undiagnosed pheochromocytoma.

III. The molecular basis for the relationship between hypertension, insulin resistance, and obesity. There is a strong interrelationship between essential hypertension, insulin resistance, and obesity. Decreased sensitivity to the action of insulin plays a major role in the pathogenesis of diabetes and has been strongly correlated with both hypertension and obesity. Dr. Quon, our new tenure-track scientist, has developed a novel method for transient transfection of DNA into rat adipose cells in primary culture. Using this model system, he has already obtained direct evidence for the roles of a number of different enzymes and substrates in the early actions of insulin. He is currently in the process of defining the promotor elements and regulatory transcription factor binding sites, using deletion mutants and point mutants of the *ob* promotor. Leptin, the *ob* gene product, is a putative satiety hormone that has recently come to national attention.

Dr. Quon will also use these techniques to elucidate insulin signal transduction pathways in vascular endothelium, especially as related to nitric oxide production. This will be done by characterizing the insulin response of human umbilical vein endothelial cells in primary culture and using the adipose cell transfection system to dissect insulin signaling pathways related to nitric oxide.

His third approach will be to identify, clone and characterize insulin-regulated genes in vascular endothelium that may participate in insulin-stimulated nitric oxide production.

MOLECULAR DISEASE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

Plasma lipids are carried in the blood by lipoproteins. Lipoproteins contain several lipids including cholesterol, phospholipids and triglycerides as well as proteins, designated apolipoproteins (apo). The overall objective of the combined basic and clinical research programs of the Molecular Disease Branch is the elucidation of the role of plasma lipoproteins in lipid transport in normal individuals and in patients with elevated plasma lipid levels who are at risk for the development of premature cardiovascular disease and other diseases including pancreatitis. A major focus of research in the Branch is the analysis of the physiological role of apolipoproteins and lipoproteins in cholesterol and triglyceride transport, and the elucidation of the pathophysiological mechanisms involved in the regulation of lipoprotein biosynthesis, transport, and catabolism in patients with disorders of lipoprotein metabolism and atherosclerosis. Studies are also conducted to establish the genetic defects in patients with elevated blood lipids in order to establish improved methods for detection of individuals at risk for heart disease or pancreatitis. The ultimate goal of this research program is the development of new methods for diagnosis as well as the advancement of more definitive approaches to therapy including gene therapy for the genetic disorders of lipid metabolism.

LIPOPROTEIN METABOLISM

High density lipoproteins

Clinical and epidemiological studies have definitively established that high density lipoproteins are inversely associated with the development of premature cardiovascular disease. The mechanism(s) by which HDL protects against cardiovascular disease is not completely understood. HDL has been proposed to play a central role in cholesterol metabolism by removing excess cholesterol from cells and transporting it to the liver where it can be removed from the body by a process which has been termed reverse cholesterol transport. Alternatively a cholesterol present in HDL maybe also transferred to very low density lipoproteins (VLDL) or low density lipoproteins (LDL) by cholesterol ester transfer protein (CETP). The cholesterol transferred from HDL to VLDL and LDL is returned to the liver by the LDL and remnant receptor pathways. Recent studies have established that plasma HDL are heterogeneous and different lipoprotein particles within HDL have been proposed to have separate physiological functions. Two important classes of lipoprotein particles within HDL are particles which contain only apoA-I (LpA-I) and particles which contain both apoA-I and apoA-II (LpA-I:A-II). LpA-I particles have been proposed to be the major anti-atherogenic lipoprotein particles within HDL. The LpA-I:A-II particles appear to offer little protection against the development of early heart disease. CETP was shown to be primarily associated with LpA-I while the preferred substrate for hepatic lipase is LpA-I:A-II. To understand the mechanisms involved in the protective effect of HDL in cardiovascular disease we have performed a series of detailed kinetic studies on LpA-I and LpA-I:A-II metabolism in controls and subjects with reduced or elevated levels of HDL cholesterol. Metabolic studies in normal volunteers have established that LpA-I:A-II is formed by the addition of apoA-II to the LpA-I particle. Kinetic studies in control subjects also established that LpA-I was catabolized at a significantly faster rate than LpA-I:A-II.

Analysis of control human plasma revealed that there are three major subclasses of LpA-I. These three subclasses, designated Large, Medium, and Small LpA-I, have different lipid and apolipoprotein composition as well as concentrations of CETP and lecithin cholesterol acyltransferase (LCAT) activities indicating the important heterogeneity of the LpA-I particles. The metabolism of the three LpA-I particles was investigated in control subjects. The catabolism of the Small LpA-I was the fastest of the three LpA-I particles clearly establishing that lipoprotein particle size as well as apolipoprotein conformation may play a pivotal role in the catabolism of LpA-I.

To gain additional information on the metabolism of LpA-I and LpA-I:A-II, kinetic studies were performed in fifty normolipidemic subjects and the associations with lipids and apolipoprotein levels determined. Triglyceride levels inversely correlated with only LpA-I. Plasma apoA-I levels were most strongly correlated with the catabolic rate. In contrast plasma apoA-II levels were only correlated with the rate of apoA-II synthesis not catabolism. Plasma LpA-I levels are correlated with the catabolism of both apoA-I and apoA-II while LpA-I:A-II levels were correlated with the synthesis rates of both apoA-I and apoA-II. Based on multiple regression analysis the production rate of apoA-II is the only independent kinetic parameter determining the plasma LpA-I levels.

A comprehensive review of subjects with hypoalphalipoproteinemia indicates that not all individuals with low HDL are at increased cardiovascular (CVD) risk. Subjects with hypoalphalipoproteinemia characterized

by low levels of HDL but not associated with markedly increased risk of CVD include LCAT deficiency, Fish Eye disease, and Tangier disease. Recently we have reported several kindreds with familial hypoalphalipoproteinemia with HDL cholesterol levels ranging from 7 to 15 mg/dl without premature cardiovascular disease. Kinetic studies in five familial hypoalphalipoproteinemic probands without CVD revealed that both LpA-I and LpA-I:A-II are rapidly catabolized leading to low HDL levels. Catabolism of LpA-I:A-II is slightly faster than LpA-I leading to a normal or increased ratio of plasma LpA-I/LpA-I:A-II.

The combined results from these and other studies have indicated that hypoalphalipoproteinemia is heterogeneous and not all patients with low HDL have an increased risk of premature CVD. Detailed cardiovascular and lipoprotein evaluation will be required to identify individuals with low HDL that have an increased CVD risk. These results provide new insights and approaches to the identification of which individuals with low HDL are at an increased risk of premature heart disease.

Lipoprotein and hepatic lipases

Lipoprotein lipase (LPL) and hepatic lipase (HL) which are attached to the capillary endothelium of peripheral tissues and the liver, respectively play a central role in lipid and lipoprotein metabolism. LPL is the principal enzyme responsible for the hydrolysis of plasma triglycerides, while HL functions primarily as a phospholipase and is a pivotal enzyme in the conversions of IDL to LDL and HDL_2 to HDL_3. Over the last several years clinical and molecular studies have been performed to increase our understanding of the structure and function of LPL and HL and to delineate the role of LPL and HL in lipid and lipoprotein metabolism in normal subjects and in patients with dyslipoproteinemias. LPL, HL, and pancreatic lipase have a high degree of structural homology and form a lipase gene family. The crystalline structure of pancreatic lipase has been elucidated and used as a molecular model for the lipase gene family. Initial studies in the Branch have utilized site-directed mutagenesis and construction of chimeric enzymes to gain insight into the structural and functional domains of LPL and HL.

LPL and HL contain a "lid" or loop of 22 amino acids containing two amphipathic helices which have been proposed to play a critical role in modulating the access of lipid substrates to the catalytic site of the enzymes. To gain insight into the structural basis for the different substrate specificities of LPL and HL in vitro and in vivo studies was undertaken using site-directed mutagenesis as well as recombinant adenoviral vectors to generate mutant lipases. Disruption of the 2 amphipathic helices present in the lipase lids results in loss of enzymic activity using both triglycerides and phospholipid (PL) substrates while esterase activity against the short-chained water soluble substrate tributyrin was preserved. Chimeric lipases containing the LPL backbone with the HL lid, the HL backbone with the LPL lid and the amino terminus of LPL with the carboxyl-terminus of HL had markedly different abilities to hydrolyze PL substrates. The presence of the LPL lid augmented triglyceride hydrolysis whereas the HL lid enhanced phospholipid hydrolysis. The in vitro studies have now been extended in vivo using recombinant adenovirus expressing either native HL , native LPL and lipases containing lid mutations. This lipase constructs were injected in HL-deficient mice which have elevated plasma levels of total cholesterol and phospholipids. Animals injected with the adenovirus containing native HL had a significant decrease in plasma PL and a marked change in lipoprotein profile; however, those injected with HL with the LPL lid had no change in plasma PL levels. Mice injected with native LPL had minimal changes in the plasma lipoproteins. However, injection of LPL containing the HL lid had a marked decrease in phospholipids and significant change in the lipoprotein profile. These studies indicate that exchange of the HL with the LPL lid or LPL with the HL lid modifies the ability of the lipases, in vivo, to hydrolyze PL and thus, established an important role of the lipase lid in mediating lipase substrate specificity. Based on this data it was concluded that the two amphipathic helices in both LPL and HP are essential for phospholipid as well as triglyceride hydrolysis. The HL lid increases a phospholipid hydrolysis whereas the LPL lid enhances triglyceride hydrolysis. Based on these combined studies we have proposed that the lipase lid plays a pivotal role in determining the substrate specificity of both LPL and HL.

GENETIC DSYLIPOPROTEINEMIAS

Elevated blood levels of LDL and reduced levels of HDL have been well established as important risk factors for the development of early heart disease. Elevated levels of plasma triglycerides have been associated with both pancreatitis and early heart disease. Over the years studies in the Branch have focused on the elucidation of the molecular and metabolic defects associated with the genetic dyslipoproteinemias. The analysis of single gene defects in patients with dyslipoproteinemias has provided major new information on the roles of transfer proteins, enzymes, and apolipoproteins in lipoprotein metabolism. Recent studies have focused on the following genetic dyslipoproteinemias.

Lecithin cholesterol acyltransferase deficiency

Lecithin cholesterol acyltransferase (LCAT) is a plasma enzyme present primarily on HDL catalyzes the esterification of plasma free cholesterol. Cholesteryl esters formed by LCAT are incorporated into the core of HDL and LDL particles. LCAT has a key role in the process termed reverse cholesterol transport in which cholesterol is removed from cells, esterified in plasma, and the cholesteryl esters are transported directly back to the liver on HDL or the cholesteryl esters are transferred to VLDL or LDL by CETP with ultimate return to the liver via the LDL and remnant receptor pathways.

Patients with structural defects in the LCAT gene present two strikingly different clinical features. In Classical LCAT deficiency the patients have severe HDL deficiency, hemolytic anemia, cloudy corneas, and progressive renal disease. In a separate clinical syndrome, Fish Eye Disease (FED), the patients have HDL deficiency, severe cloudy corneas, but no renal disease or hemolytic anemia. During the last several years our Branch has systematically determined the molecular defects in the LCAT gene in probands with both Classical LCAT deficiency and FED. Based on the combined results from these studies we have established that the reason for the difference in the two clinical syndromes associated with LCAT deficiency is the residual activity of the mutant LCAT enzyme. Approximately 10 to 20% residual activity of the mutant LCAT enzyme appears to be sufficient activity to prevent the renal disease and hemolytic anemia. The plasma of LCAT deficient patients is a unique source of newly synthesized or nascent HDL particles. It has been proposed that nascent HDL are the initial acceptor of cellular cholesterol and are the most effective particles in removing excess cholesterol from cells. Studies were undertaken to isolate and characterize these nascent HDL particles from LCAT deficient patients. Using a combination of ultracentrifugation, and affinity chromatography homogeneous nascent HDL particles have been isolated and are currently being used in kinetic studies to analyze the metabolism of newly synthesized nascent HDL particles. These studies will provide major new data on HDL metabolism in control subjects and patients with genetic dyslipoproteinemias.

Familial hypercholesterolemia

Patients homozygous for familial hypercholesterolemia (FH) manifest profound hypercholesterolemia, xanthomas, and cholesterol deposition in a variety of tissues including the eye, tendons, and arterial wall. These patients have accelerated atherosclerosis and may develop symptomatic CVD from the ages of 2-30 years, and many die before the age of 20. The cause for the 10 fold increases in total and LDL cholesterol concentrations is a defect in the plasma clearance of cholesterol-rich LDL due to a structural defect in the LDL receptor. More than 150 mutations in the LDL receptor gene can lead to the loss of the expression of functional LDL receptors on the surface of liver cells. We have previously established that the degree of LDL receptor dysfunction determined on cultured skin fibroblasts from these patients is highly correlated with the concentrations of plasma LDL cholesterol. Over the years we have applied a variety of therapies to reduce the LDL cholesterol concentrations in these patients including diet, combination hypolipidemic drug therapy, portacaval shunting of the liver, plasma exchange, LDL apheresis, liver transplantation, and most recently, adenoviral gene therapy. The degree of coronary artery atherosclerosis and the response to lipid-lowering intervention is variable among the patients with LDL receptor defects. Prospective studies have been performed on the rate of progression of atherosclerosis by both invasive and noninvasive techniques in FH patients. We have assessed the extent and severity of both cholesterol deposition into tissues and in atherosclerosis using computerized axial tomography. This noninvasive test led to a new concept in atherosclerotic cardiovascular disease risk assessment, the cholesterol-years risk score. The results of the analysis of patients with familial hypercholesterolemia have theoretic as well as practical implications for diagnosis and therapy in these patients as well as in individuals with more common forms of atherosclerosis.

ApoA-I deficiency

ApoA-I, the major structural apolipoprotein of LpA-I, has been proposed to be the major apolipoprotein involved in transport of excess cellular cholesterol back to the liver. As discussed above genetic dyslipoproteinemias with mutations in single genes have provided major insights into the factors which modulate plasma HDL levels and metabolism. To date only 4 kindreds with apoA-I deficiency has been extensively studied. Not all of the probands with apoA-I deficiency have an increased risk of early heart disease and the reason for the lack of vascular disease in these probands is as yet unexplained. We have recently evaluated a 29 year old male with corneal opacification, xanthelasma, and planar xanthomas. Plasma lipoproteins were remarkable for the very low level of HDL cholesterol, presence of LpA-II particles, and the absence of plasma apoA-I. The proband has a 62 year old aunt with established cardiovascular disease and no plasma apoA-I.

The molecular defect in the apoA-I gene was evaluated and shown to be a single base insertion resulting in a nonfunctional truncated apoA-I. Detailed kinetic studies revealed the LpA-II particles were rapidly catabolized when compared to LpA-I and LpA-I:A-II in control subjects. The proband was entirely symptomatic with no symptoms of cardiovascular disease. Of particular interest, however, was the finding of significant aortic calcification establishing that the proband had significant premature cardiovascular disease. The combined results from this study established that the complete absence of plasma apoA-I results in marked HDL deficiency and premature cardiovascular disease. In the absense of apoA-I, LpA-II may not serve a protective role. In addition noninvasive screening of patients with apoA-I deficiency as well as other low HDL syndromes by ultrafast CT will permit the identification of premature atherosclerosis in otherwise asymptomatic patients who should be considered for treatment of their dyslipoproteinemia.

TRANSGENIC ANIMAL MODELS OF LIPOPROTEIN METABOLISM

The development of the methodology for overexpressing specific genes in transgenic animals provides the unique opportunity to study the role of specific genes important in lipoprotein metabolism and the development of atherosclerosis. Transgenic mice and rabbits overexpressing several different genes have been developed in the Branch.

A. Transgenic mice models

LCAT transgenic mice

In order to gain additional insights into the role of LCAT in reverse cholesterol transport and HDL metabolism, transgenic mice were developed which over expressed human LCAT (hLCAT) at plasma levels 10,14 and 100 fold higher than control mice. The transgene was expressed virtually only in the liver. Compared to 24 age and sex-matched siblings, transgenic mice (% of control) had elevated plasma cholesterol (133-237%), CE (141-267%) and HDL-C (123-209%) but similar plasma levels of triglycerides, phospholipids, apoB-containing lipoproteins, apoA-I and apoA-II. FPLC analysis of hLCAT transgenic mouse plasma revealed larger sized HDL particles enriched in cholesteryl esters and phospholipids.

The effects of LCAT on diet induced hyperlipidemia were analyzed in age/sex matched LCAT transgenic and control animals on a high cholesterol-fat diet for 21 days. On the high cholesterol-fat diet transgenic mice had significantly higher ($p<0.05$) HDL-C as well as reduced TC/HDL ratios than controls, with no significant differences in triglycerides, phospholipids, cholesteryl esters, LCAT mass and activity. FPLC analysis of transgenic mouse plasma revealed significant increases in HDL-C, cholesteryl esters and phospholipids with reciprocal decreases in IDL/LDL-C, cholesteryl esters and phospholipids. These results established that LCAT overexpression decreased the dietary induced increase in the atherogenic IDL/LDL particles.

In order to evaluate the effect of overexpression of the human LCAT gene on diet-induced atherosclerosis, control and LCAT transgenic C57B mice were sacrificed 16 weeks after initiation of a high fat-diet. Analysis of aortic lesions revealed no differences in the mean lesions size between control and transgenic mice on the high-fat diet. These findings indicate that in the mouse model, LCAT overexpression does not protect against the development of diet-induced atherosclerosis. Thus, LCAT modulation of plasma HDL concentration does not confer protection from atherosclerosis in this animal model.

The availability of both transgenic animals and adenovirus vectors expressing specific genes has permitted the initiation of studies to evaluate the effects of gene-gene interactions in HDL metabolism. LCAT, CETP and HL gene-gene interactions were analyzed, in vivo, utilizing recombinant adenovirus to express human HL and CETP in transgenic mice overexpressing human LCAT at plasma levels 100 fold higher than control mice. LCAT transgenic mice have increased plasma cholesterol, cholesteryl esters and HDL-C concentrations as well as marked HDL heterogeneity not present in control animals. Unlike humans, mice are deficient in CETP activity and thus, in these animals CETP-mediated lipid exchange between HDL and apoB containing lipoproteins does not occur.

Adenovirus-mediated expression of HL in control and LCAT transgenic mice resulted in approximately a 50-60% reduction in plasma cholesterol, phospholipids and HDL in both groups. Compared to control animals, HL expression in transgenic mice resulted in a preferential hydrolysis of the LpE-rich HDL as well as normalization of the heterogeneous HDL profile thus indicating the coordinate role for these two enzymes in modulating HDL particle heterogeneity.

Adenovirus-mediated expression of CETP in control and LCAT transgenic mice resulted in increased plasma apoB lipoproteins as well as decreased HDL concentrations, a lipoprotein pattern reminiscent of human

profiles which indicates CETP-mediated effective transfer of cholesteryl esters from HDL. Compared to controls, expression of CETP in LCAT transgenic mice resulted in a more protective lipoprotein profile, thus establishing the coordinate role of CETP and LCAT in HDL metabolism.

ApoA-II transgenic mice

The function of apoA-II in HDL metabolism has been assessed by the study of mice overexpressing apoA-II. ApoA-II transgenic mice have elevated plasma triglyceride levels, increased HDL levels, increased apoA-I as well as apoA-II levels, and the production of LpE particles. ApoA-I and apoA-II kinetic studies were performed in mice overexpressing mouse apoA-II and age matched controls. In apoA-II transgenic mice both apoA-I and apoA-II were catabolized slower than apoA-I and apoA-II in control mice. With progressive increases in plasma apoA-II levels there was a parallel increase in plasma triglycerides and IDL sized lipoproteins containing two major lipoprotein particles, LpB and LpA-II:E. The triglyceride-rich lipoproteins had virtually no apoE by FPLC analysis.

These studies showed that the catabolism of apoA-I and apoA-II was slower in the apoA-II overexpressors compared to the controls. The increased HDL level in the mice overexpressing apoA-II is due to both an increased synthesis and decreased catabolism of HDL.

B. Transgenic rabbit model

A transgenic rabbit program has been developed in the Branch to systematically investigate the impact of genes relevant to HDL as well as LDL metabolism, reverse cholesterol transport, and atherosclerosis in the rabbit animal model. The rabbit model compliments the mouse model in that the rabbit does not synthesize apoA-II, has partial hepatic lipase deficiency, high plasma CETP activity, and atherosclerotic lesions that are similar to man. The metabolic differences between rabbits and mice provide the opportunity to study the effects of specific genes on lipoprotein metabolism and atherosclerosis in two separate model systems. New methods necessary for establishing the rabbit transgenic program included the development of superovulation strategies, mating and breeding techniques, and specific apolipoprotein assays.

ApoA-I transgenic rabbits

Initial studies were undertaken to examine the effects of overexpression of apoA-I and increased HDL levels in a LDL receptor deficient rabbit with marked hyperlipidemia and atherosclerosis. This strain of rabbit, designated the Watanabe Heritable Hyperlipoproteinemic (WHHL) rabbit, provides a system to test a variety of hypotheses relevant to lipoprotein metabolism and atherosclerosis. To date 14 founder transgenic rabbits expressing apolipoprotein A-I have been identified. The impacts of overexpression of apolipoprotein A-I on both lipoprotein metabolism and the ability of HDL to prevent atherosclerotic cardiovascular disease are being pursued.

LCAT transgenic rabbits

The effect of overexpression of LCAT on plasma lipoprotein metabolism and atherosclerosis in the rabbit has been initiated. Three founder lines of rabbits have been identified which have integrated and expressed the LCAT gene. The total and high density lipoprotein cholesterol and apolipoprotein A-I concentrations are increased 2-3 fold that of controls. In addition, there is a marked reduction in the concentration of the apoB containing particles, VLDL, IDL, and LDL. Kinetic studies revealed that the increase in plasma apoA-I and HDL cholesterol is due to a decreased catabolic rate of apoA-I leading to hyperalphalipoproteinemia. The level of expression of the LCAT enzyme is positively correlated with the plasma apoA-I and HDL cholesterol levels. Rabbits with high, intermediate, and low levels of plasma LCAT expression have parallel decreases in the fractional catabolic rates of plasma apoA-I. Nontransgenic rabbits fed a high cholesterol diet have a marked increase in the concentration of the atherogenic very low, intermediate, and low density lipoproteins. In contrast, LCAT transgenic rabbits have substantially higher concentrations of HDL and lower concentrations of the atherogenic VLDL, IDL, and LDL. The effect of LCAT overexpression on diet induced atherosclerosis in these transgenic rabbits is currently being determined.

GENE THERAPY

The ultimate correction of the molecular defects in patients with the genetic dyslipoproteinemias will be greatly facilitated by the development of gene therapy technology. In order to establish the safety and efficacy of this approach we have initiated a gene therapy program for the correction of gene defects in mice

in which the function of a specific gene has been inactivated by the use of homologous recombination. These gene knock out mice models provide a unique opportunity to study the correction of gene defects by gene therapy.

A. ApoE Deficient Mice

ApoE is present in VLDL, IDL and HDL and plays a pivotal role in the metabolism of plasma lipoproteins by functioning as a ligand for the LDL and remnant receptors facilitating the clearance of remnant particles from the circulation. Patients with a defect in the apoE gene resulting in apoE deficiency develop type III hyperlipoproteinemia and premature atherosclerosis. Recently, apoE deficient mice with marked hypercholesterolemia and spontaneous atherosclerosis have been developed by homologous recombination. These animals are a useful model for evaluating the potential for gene therapy of an apolipoprotein defect which results in a genetic dyslipoproteinemia. We have generated a recombinant adenoviral vector containing human apoE cDNA (apoE rAdV) for replacement of apoE in apoE deficient mice. The lipoprotein profile of the apoE deficient mice included plasma cholesterol of 609±108 mg/dl, triglycerides of 101±50 mg/dl, and cholesterol-rich VLDL/IDL. After IV infusion of the apoE rAdv, apoE-deficient mice had peak (day 6) plasma human apoE levels ranging from 2.3 to 648 mg/dl. Following apoE replacement the plasma lipids and lipoproteins were normalized to control values with virtual complete absence of cholesterol rich VLDL/IDL and the generation of HDL. ApoE expression and normal plasma lipids were maintained for a period of 4 weeks after apoE rAdv injection. Analysis of aortic atherosclerotic lesions in apoE deficient mice at 4 weeks after apoE rAdv administration revealed marked reduction in atherosclerosis when compared to an animal injected with the control luciferase adenovirus. Thus, expression of physiologic concentrations of apoE for 1 month normalized plasma lipids and lipoproteins and resulted in marked reduction in aortic atherosclerosis. Successful replacement of apoE in apoE deficient mice demonstrates the feasibility of gene therapy for human genetic apolipoprotein deficiencies.

B. Hepatic Lipase Deficiency

Hepatic lipase (HL) is an endothelial bound enzyme which mediates the hydrolysis of triglycerides and phospholipids present in IDL and HDL. Patients with HL deficiency have increased IDL and HDL and have an increased risk of premature cardiovascular disease. In order to evaluate the feasibility of replacing an endothelial bound lipolytic enzyme we have utilized a recombinant adenovirus containing the human hepatic lipase cDNA (HL rAdv) and a knock out mouse model with HL deficiency. HL deficient mice have elevated plasma cholesterol, phospholipids, and free cholesterol when compared to age/sex matched control mice. The FPLC profile of HL deficient mice demonstrated a marked increase in HDL-C and phospholipid content relative to control mice. Hepatic lipase deficient mice injected with HL rAdv had a peak (day 4- 5) reduction in cholesterol(-23%, $p<0.001$), phospholipids (-30%, $p<0.002$) and free cholesterol (-35%, $p<0.05$). Immunoblot analysis of mouse post-heparin plasma 4 days post-infusion of HL lipase rAdv demonstrated the presence of HL lipase. Plasma analysis on FPLC showed dramatic decreases in HDL-C and phospholipids as well as the formation of LDL-sized cholesterol and phospholipid enriched particles in rAdv treated mice. Virtually all expressed HL was detected in post-heparin plasma indicating that the enzyme had attached to the capillary endothelium. These combined results establish that gene replacement using recombinant adenovirus vectors can be used to successfully replace an endothelial bound lipolytic enzyme with normalization of the lipoprotein phenotype. Future development of improved vectors with longer expression will permit the initiation of gene therapy for enzyme deficiencies in man.

Annual Report of the Molecular Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1994 - September 30, 1995

This report summarizes the activities of the Section on RNA and Protein Biosynthesis, the Unit on Adeno-Associated Virus (AAV) Molecular Biology and Gene Transfer, and the Unit on Chromatin and Gene Expression. Emphasis is directed towards the acquisition of basic knowledge and technology required for successful gene therapy.

SECTION ON RNA AND PROTEIN BIOSYNTHESIS

This section studies the mechanisms which regulate expression of the subunits of the translation initiation factor eIF-2, at both the transcriptional and translational levels. In addition, the targeted delivery of recombinant adeno-associated viral vectors for the gene therapy of hemophilia B is being developed.

During the past year this section has achieved the following:

1. By RT-PCR, both the sense and antisense eIF-2α transcripts have been identified. In G_o cells, formation of dsRNA results, leading to the rapid degradation of both transcripts.

2. Upon mitogenic activation, transcription from the antisense promoter is inhibited while sense transcription is increased. This allows normal processing and accumulation of eIF-2α mRNA.

3. Cloning and sequencing of the cDNA for the 4.2 kb mRNA of eIF-2 has revealed the presence of four polyadenylation sites. In humans only two sites are recognized. Although both mRNAs are translated with equal efficiency the stability of the 4.2 kb mRNA is greater than that of the 1.6 kb mRNA.

4. The induction of protein synthesis immediately after T cell activation is mediated by the PMA pathway and not through the ionomycin pathway.

5. cDNA encoding α-PAL, a novel transcription factor which regulates eIF-2α transcription, has been transcribed and translated. By EMSA, α-PAL DNA binding activity was shown to be increased 5-10 fold by phosphorylation.

6. α-PAL kinase activity is stimulated following mitogenic activation of G_o T-cells.

7. Dephosphorylation of the gamma subunit of eIF-2B during T-cell activation is responsible for the rapid onset of translational activity during mitogenic

stimulation of T-cells.

8. Using microsurgical techniques, recombinant AAV vectors expressing β-galactosidase have been targeted to specific regions of the hepato-biliary tree.

UNIT ON AAV MOLECULAR BIOLOGY AND GENE TRANSFER

This unit studies the molecular biology of adeno-associated virus (AAV) and its potential use as a gene therapy vector. During the past year:

1. A new packaging system which can generate over 1000 recombinant AAV particles from each producer cell has been developed. This procedure may allow production of sufficient quantities of rAAV for animal protocols.

2. Purification of the rAAV has been achieved by cation exchange chromatography.

3. A Rep responsive human ori (origin of replication) has been identified.

4. DNA synthesis has been reconstituted in vitro using oligonucleotides containing the Rep binding and nicking sites, pol delta and PCNA.

5. The canonical Rep binding sequence has been determined using a pool of random oligonucleotide probes.

6. Deletion analysis of Rep 78 identifies the carboxy terminus as being involved in the regulation of gene expression.

7. These results have enabled us to propose a model for Rep-dependent site-specific integration.

UNIT ON CHROMATIN AND GENE EXPRESSION

This unit studies the regulation of gene expression mediated by altered chromatin structure. During the past year the following has been achieved:

1. The core element of the chicken beta-globin insulator was identified.

2. Protein factors that bind the core element were characterized.

3. "Super" insulator was created by multimerizing the insulator core element.

4. It was demonstrated that although the chicken beta-globin insulator works in Drosophila, a Drosophila insulator, scs, does not work in vertebrate cells.

ANNUAL REPORT OF
THE PULMONARY-CRITICAL CARE MEDICINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1994 Through September 30, 1995

The Pulmonary-Critical Care Medicine Branch (P-CCMB) has initiated a series of research programs aimed at defining the pathophysiology of acute and chronic lung disease. With the merger of the Laboratory of Cellular Metabolism and the Pulmonary Branch, the P-CCMB acquired a strong core of investigators interested in signal transduction, specifically related to guanine nucleotide-binding proteins, cyclic nucleotide phosphodiesterases, and ADP-ribosylation. To initiate clinical studies related to pulmonary disease, the Branch expanded its interests to include nitric oxide, lymphangioleiomyomatosis (LAM), hyperoxia, and α1-antitrypsin deficiency. Under the new Clinical Studies Section, clinical research protocols were initiated in the above areas as well as in areas related to the ongoing basic research in cyclic nucleotide phosphodiesterases, guanine nucleotide-binding proteins, and ADP-ribosylation. With addition of Dr. Kolobow's Section to the Branch, expertise was added in the design of cardiopulmonary devices and the optimal management of assisted ventilation.

Role of Nitric Oxide in the Pathophysiology of Lung Disease

Nitric oxide (NO) is a small free radical that is synthesized from L-arginine by nitric oxide synthase (NOS) and is increasingly recognized as a major control point in physiological and pathophysiological processes. The lung may be affected by endogenous and/or exogenous (e.g., from air pollutants, cigarette smoke) NO, leading to acute and chronic lung injury. Since the lung is an oxygen-rich environment, NO may react with oxygen-derived molecules, generating highly reactive compounds (e.g., peroxynitrite). This project is aimed at evaluating the role of NO in the pathogenesis of lung disease.

Initial studies looked at the enzymes involved in the synthesis of NO in the lung. Three isoforms of NOS have been identified; two subtypes are constitutive enzymes found in endothelium and neurons, respectively, whereas the other is an inducible enzyme (iNOS) (e.g., by cytokines), found in many types of cells. iNOS, the critical enzyme responsible for the enhanced synthesis of NO in inflammatory states, is a predominant isoform in the lung and is abundantly expressed in epithelial cells. To elucidate the regulation of NO synthesis in the lung, the transcription initiation sites and structure of the 5′-untranslated region (5′-UTR) of the human iNOS gene were examined in lung-derived cultured cell lines, and in lung samples obtained from normal subjects. Consistent with previous reports, a predominant transcription initiation site was located 30 bp downstream of the first nucleotide of the TATA box. However, despite the presence of a TATA box, multiple transcription initiation sites were observed, some extending several hundred base pairs upstream from the main TATA-directed initiation site. Alternative splicing in the 5′-UTR that included a deletion of exon 1 plus the adjacent 35 bp of the 5′ flanking region (-1 to -35) resulted in further diversity. Although the majority of iNOS mRNA transcripts were initiated as expected in a TATA-containing gene, a small proportion of iNOS mRNAs were apparently TATA-independent. They too were up-regulated by cytokines (interferon-γ, interleukin-1β,

tumor necrosis factor-α, and interleukin-6). The latter observations are consistent with the behavior of many TATA-less genes and with the presence in the iNOS gene of multiple transcription element consensus sequences involved in cytokine-stimulated gene expression. The study also demonstrated that in the absence of cytokines, normal human bronchial epithelial cells, other epithelial cells (e.g., DLD-1 cells), and to lesser extent, normal human alveolar macrophages, contained significant amounts of iNOS mRNA, suggesting the cells may be under chronic stimulation, perhaps by environmental factors. Elucidation of the molecular mechanisms involved in iNOS regulation could potentially lead to therapeutic intervention.

Clinical and Basic Studies on α1-Antitrypsin Deficiency

Chronic obstructive pulmonary disease (COPD) is the fifth leading of cause of death in the US. Patients with emphysema represent a significant proportion of individuals with COPD; α1-antitrypsin (α1AT) deficiency, a common genetic disease, affecting an estimated 40,000-70,000 Americans, represents a mono-genetic model of emphysema. Although there are several mutations that may be responsible for α1AT deficiency, in the majority of α1AT-deficient individuals (who are PI*Z), a single amino acid change causes accumulation of the mutant protein within the cell and a 5-fold reduction in secreted α1AT. Our objectives are to characterize the basic mechanisms and clinical consequences of α1AT deficiency and to develop treatment strategies based on these observations.

The multi-center α1AT Deficiency National Registry has made some important new observations regarding the clinical characteristics of α1AT deficiency: 1) reactive airways is a marker for the rapid decline in lung function; 2) non-transplanted individuals survive longer than those who receive transplanted lungs; and 3) among individuals with lung disease, ex-smokers have the same rate of decline in lung function as those who have never smoked.

In evaluating the molecular mechanisms of novel α1AT null variants (variants associated with the complete absence of α1AT), it was determined that different mechanisms can be associated with the null phenotype. Additionally, it was found that the profound reduction in secreted Z α1AT resulted from degradation, rather than retention, of the Z protein within the cell. Furthermore, degradation of the Z protein appeared to be caused by an energy-dependent, short lived, non-lysosomal protease. When degradation of α1AT was pharmacologically inhibited, movement to the Golgi and secretion of the Z protein was similar to that of normal α1AT.

Regulation of Vesicular Trafficking by ADP-ribosylation factors

ADP-ribosylation factors (ARF), a multigene family of 20-kDa guanine nucleotide-binding proteins, were initially discovered as activators of cholera toxin ADP-ribosyltransferase activity, and subsequently shown to be critical components of the vesicular trafficking system, which is crucial for both regulated and constitutive secretion in the lung and other organs. Current ongoing studies examine the function of ARFs and associated proteins in vesicular trafficking.

Guanine nucleotide-binding (G) proteins in other systems appear to serve as an intermediary between a receptor, which accelerates exchange of GDP for GTP, on the G protein, thus promoting activation, and an effector, which is the target of the activated G protein. A possible effector for ARF is phospholipase D, an enzyme that catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. In studies

performed in collaboration with Dr. Sue Goo Rhee, it was shown that all members of the ARF gene family activated phospholipase D, whereas the ARF-like proteins, or ARLs, possessed very little activity. Recombinant ARFs appeared to be more active when myristoylated at their amino termini, a modification that was shown previously to promote their interaction with Golgi and other membranes.

It appeared that ARFs, but not ARLs, are capable of activating both phospholipase D and cholera toxin. To determine which regions of the proteins are responsible for the functional differences between ARF and ARL (which differ in only 78 of 181 amino acids), recombinant chimeric ARF-ARL proteins were examined for their ability to activate phospholipase D and cholera toxin. The data are compatible with the hypothesis that the amino third of ARF is essential for phospholipase D activation whereas the carboxy two-thirds contains the cholera toxin activation domain.

The amino terminal domain appears to be crucial, in part due to its myristoylation, for ARF binding to membranes. As a mutant ARF1 protein, lacking the first thirteen amino acids, activated cholera toxin, it appeared that the amino terminus was not required. The mutant remained active after removal of bound nucleotide, even in the absence of GTP, under assay conditions that did not destabilize the protein (i.e., in the absence of detergent), suggesting that nucleotide was not required for the protein to assume an active conformation. These observations stand in contrast to those with other GTP-binding proteins which always appear to be nucleotide dependent.

ARF is active with GTP bound and inactive with bound GDP. Activation of ARF-GDP is enhanced in the presence of a guanine nucleotide-exchange protein (GEP). Prior studies demonstrated that a 700-kDa complex, containing GEP, was sensitive to inhibition by brefeldin A (BFA). The purified GEP, a monomeric protein of 60 kDa, was stimulated by phospholipids and insensitive to BFA, suggesting that the BFA sensitivity factor had been removed during purification. Moreover, the purified GEP was selective in its preferential activation of the Class I ARFs (ARFs 1 and 3), suggesting that multiple GEP-like proteins may be required for activation of the different ARFs.

The conversion of activated ARF-GTP to the inactive state appears to be facilitated by a specific GTPase-activating protein or GAP, as documented by studies from the Cassel and Kahn laboratories. Preliminary studies in our laboratory suggest that an ARF-GAP is capable of converting ARF-GTP from all three classes to the inactive state containing bound GDP. With the isolation of GEP and GAP, the proteins required for the conversion of ARF from active to inactive state and back again, can now be studied with regard to their regulatory potential in the initiation and termination of discrete steps in vesicular trafficking.

Regulation of ADP-ribosylation in Mammalian Cells

ADP-ribosylation is critical to the action of numerous bacterial toxins involved in the pathogenesis of disease (e.g., pertussis toxin, cholera toxin). Mammalian and some other eukaryotic species possess ADP-ribosyltransferases that catalyze reactions similar to those catalyzed by several of the toxins. Prior studies coupled a subfamily of the mammalian and avian ADP-ribosyltransferases, those catalyzing the ADP-ribosylation of arginine residues in proteins, with ADP-ribosylarginine hydrolases, enzymes that cleave the ribose-arginine linkage, regenerating free arginine. These two enzymes could form the basis for an ADP-ribosylation cycle, with the transferases and hydrolases participat-

ing in the opposing arms of the cycle.

Of interest with regard to the possible function of the ADP-ribosyltransferases, is the finding that an enzyme in cardiac and skeletal muscle is linked to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. Compatible with this observation is the finding that the deduced amino acid sequences from the cloned human and rabbit genes possess hydrophobic amino and carboxy terminal signal sequences. To investigate the possible regulatory roles for this enzyme, cell surface substrates for the GPI-linked protein were identified by incubation of cultured myotubes with [32P]NAD. The muscle transferase modified at least two different sites in the extracellular domain of integrin $\alpha 7$, a protein believed to participate in cell adhesion to laminin, and possibly to act through an intracellular tyrosine phosphorylation cascade. The role of ADP-ribosylation in modulating integrin-mediated signalling is under investigation.

The fate of an ADP-ribose moiety linked to integrin $\alpha 7$ was examined, as it was apparent that the intracellular location of a cytosolic ADP-ribosylarginine hydrolase would likely preclude the participation of ADP-ribose-$\alpha 7$ in a standard ADP-ribosylation cycle. In fact, the ADP-ribosylated integrin $\alpha 7$ was metabolized by a cell surface pyrophosphatase, that cleaved the ADP-ribose, and, following the action of a phosphatase, resulted in generation of ribosyl(arginine)protein, which appeared to be relatively stable. The presence of a ribose moiety on the arginine would presumably block its availability as an acceptor in another ADP-ribosylation reaction.

The muscle ADP-ribosyltransferase appears to have a counterpart in lymphoid cells, which may be involved in the regulation of the immune response. In all likelihood, substrates for the lymphocyte enzymes differ from those in muscle. Current efforts are directed at determining the structure, role and regulation of these enzymes, and their place in diseases characterized by a lymphocytic alveolitis.

Cyclic Nucleotide Phosphodiesterases (PDEs)

PDEs hydrolyze, and thereby are critical regulators of biological responses mediated by, cAMP and cGMP. Seven different PDE gene families have been identified. We have focussed on Type III cGMP-inhibited (cGI) PDEs, which are characterized by a wide tissue distribution, high affinities for both cAMP and cGMP, specific inhibition by drugs that increase myocardial contractility and relax airway and vascular smooth muscle, and rapid activation by hormones such as insulin and agents that increase cAMP.

cDNAs encoding representatives of two distinct cGI PDE subfamilies were cloned. cGIP1 and cGIP2 isoforms from different species are more closely related than are cGIP1 and 2 from the same species; they are products of distinct but related genes, on human chromosomes 11 and 12, respectively. In collaborative studies, the gene for human(H)cGIP1 has been cloned; it contains 16 exons spanning >100 kb. A mouse(M)cGIP1 cDNA and a genomic clone containing putative mouse(M)cGIP1 Exon 1 are very similar to rat(R) and (H)cGIP1 cDNA and human(H)cGIP1 Exon 1. Based on Northern blots and in situ hybridization studies, RcGIP1 and RcGIP2 mRNAs are expressed in different tissues, consistent with different functional roles for RcGIP1 and 2.

The domain organizations of cGIP1 and 2 are similar, with predicted N-terminal region regulatory domains and the catalytic domain, conserved among all PDEs, in the C-terminal region. The deduced sequences of cGIP1 and 2 differ substantially in the N-terminal portion, but are very similar in the catalytic domains except for an insertion of

44 amino acids, which is not found in the conserved domains of other PDE families and thus may not only be unique to cGI PDEs but may also identify individual cGI PDE isoforms. Active, truncated RcGIP1 and HcGIP2 recombinants lacking most of the divergent N-terminal regions exhibited high affinities for cAMP and cGMP and were sensitive to inhibition by the Type III PDE inhibitor cilostamide. HcGIP2 was, however, more sensitive to cGMP than was RcGIP1, suggesting that the 44-amino acid insertion might regulate cGMP interactions with different cGI PDE isoforms.

Deduced amino acid sequences of the N-terminal regions predict regulatory functions, including consensus cAMP-dependent protein kinase (cA-PrK) phosphorylation sites and putative hydrophobic membrane-association domains. In collaborative projects, serine 427 in RcGIP1 solubilized from adipocyte membranes was phosphorylated by cA-PrK. RcGIP1 and HcGIP2 deletion mutant recombinants (expressed in Sf9 cells) that included any portion of the N-terminal hydrophobic domain were recovered primarily in particulate fractions, whereas recombinants in which the N-terminal half of RcGIP1 or HcGIP2 was deleted were primarily cytosolic, suggesting that the hydrophobic domain is important in membrane association. Increased understanding of cellular regulation of specific PDE gene families and relationships to other PDEs will be of increasing importance in targeting inhibitors to specific PDE isoforms to alter cyclic nucleotide signalling pathways in treatment of pulmonary disorders, especially those relating to airway reactivity, inflammation, and allergy.

Pulmonary and Cardiac Assist Devices

The Section of Pulmonary and Cardiac Assist Devices has continued to explore techniques and devices to support acutely failing lungs, or heart. It was considered important to refrain from methods that exceed normal physiologic conditions by, for example, limiting peak airway pressures at the mechanical ventilator to within the normal pressure range, even in acute respiratory failure; and similarly, to keep left ventricular filling pressure within normal range with any technique of cardiac assist.

We had shown that just 12½% of total lung can sustain adequate gas exchange, provided tidal volume is reduced proportionately. We had also shown that healthy lungs ventilated at a peak respiratory pressure (PIP) of 30 cm H_2O develop significant functional abnormalities. At even higher PIP of 50 cm H_2O those alterations lead to acute respiratory failure (ARF) and adult respiratory distress syndrome (ARDS) with pulmonary histologic findings indistinguishable from those seen in ARF and ARDS in man.

The Reverse Thrust Catheter (RTC) greatly reduces dead space ventilation, and permits respiratory rates much greater than those commonly used. In the RTC designed for Intratracheal Pulmonary Ventilation (ITPV), fresh air/oxygen is delivered from the tip of the tracheal tube directly to the lungs, bypassing major upper airway dead space. Because of the design of the RTC catheter, expiration is active, and auto-PEEP (positive end expiratory pressure) is effectively avoided, even at respiratory rates of 120/min. In healthy sheep, a tidal volume (VT) as low as 1 ml/kg was well tolerated, with no impairment in gas exchange, while peak respiratory pressure (PIP) was but 1 - 2 cm H_2O above positive end-expiratory presssure (PEEP).

We have now shown in two sheep models of severe, acute respiratory failure (barotrauma and oleic acid infusion) that mechanical ventilation (MV) can be sustained at peak respiratory pressures (PIP) less than 20 cm H_2O, at rates up to 120/min. The

hemodynamic abnormalities commonly observed with conventional MV at high PIP were greatly reduced, or absent. There was massive spontaneous diuresis immediately upon initiating ITPV. Similar results were obtained when sheep were sustained on a unique, high compliance, continuous positive airway pressure (CPAP)-ITPV system, without use of MV, with tidal volumes as low as 2 ml/kg. In effect, we sustained excellent gas exchange at a PIP well below the pulmonary pressure/volume "inflection" point, indicative of optimal regional ventilation/perfusion in an acutely diseased lung.

We have shown that in the ITPV-RTC system, mucus transport is "active" and there is no need for tracheal suction, routinely, or otherwise. In vitro studies showed that mucus is physically transported outward, against gravity, by the emerging gas flow, and collects in the water trap. This process is facilitated by water droplets entrained in the air/oxygen passing through the humidifier, all of which is expelled with entrained mucus.

We have developed a new generation of thin-walled, low resistance endotracheal tubes with a unique seal at the level of the glottic opening, consisting of thin concentric "gills" with no inflatable cuff. Long-term animal studies have shown the absence of tracheal lesions (there being no inflatable tracheal cuff); glottic abnormalities were similar to or less than those observed with conventional tracheal tubes, and there was an absence of aspiration. With airway resistance 4 - 9 fold lower than conventional tracheal tubes, these new tubes are highly suitable for applications that require patient cooperation, such as CPAP.

Our animal studies with acute respiratory failure (ARF) lead us to believe that ITPV-RTC is readily applicable to clinical field usage, particularly so because results were equally good with the CPAP-ITPV system, dispensing with the use of MV altogether. The self-cleaning features of the ITPV-RTC system reduce/eliminate the need for tracheal suctioning, and reduce the likelihood of nosocomial infections.

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1994 THROUGH SEPTEMBER 30, 1995

NK-2 Homeobobox Gene. During the past year we have continued to study the *NK-2* homeobox gene of *Drosophila*. Three peptides were synthesized that correspond to predicted antigenic amino acid sequences in different regions of NK-2 protein. Three affinity purified rabbit antibody preparations were obtained, each directed against a different NK-2 peptide. Western analysis showed that antibody-1 recognizes two bands of protein from *Drosophila* embryos, one band of protein with an Mr similar to that expected for NK-2, the second, with a slightly higher Mr. Antibodies 2 and 3 bind to a 77 amino acid residue protein that contains the NK-2 homeodomain, but not to full length NK-2 protein. The distribution of NK-2 protein in *Drosophila* embryos, determined with antibody-1, was found to be similar to the distribution of NK-2 mRNA. However, the earliest expression of NK-2 protein detected was in stage 6 embryos, approximately 1 hour after the initial appearance of NK-2 mRNA. NK-2 protein was found in 2 medial clusters of neuroblasts per hemisegment, which confirms part of the pattern found for NK-2 mRNA, but NK-2 protein was not detected in lateral neuroblasts that contain relatively low levels of NK-2 mRNA.

Recently, other investigators have shown that the *ventral nervous system defective* gene (*vnd*) and the *NK-2* gene are identical. This finding and recent reports that show that *vnd* activates the proneural genes of the *achaete-scute* complex of genes (*achaete, scute,* and *lethal of scute*), which encode basic helix-loop-helix transcriptional regulatory proteins that in turn activate proepidermal helix-loop-helix genes in the *Enhancer of split (E(spl))* complex of genes that are required for the epidermal pathway of development. These results suggest that NK-2 protein is required for commitment of some cells to the neural pathway of development. About 25 percent of the neuroblasts that give rise to the ventral nerve cord are missing in null *vnd* mutants. We find that mutation of *Delta* or deletion of the *E(spl)* complex of genes results in embryos with NK-2 positive neuroectodermal cells and neuroblasts from right and left sides fused at the ventral midline. No mesectodermal cells were detected. Overproduction of NK-2 positive thoracic neuroblasts and aberrant patterns of NK-2 positive neuroblasts and neurons also were found compared to wild-type embryos. These results suggest that lateral inhibition, i.e., selection of neuroectodermal cells that segregate as neuroblasts, may involve repression of the *NK-2* gene by helix-loop-helix proteins encoded by genes in the *E(spl)* complex.

Twenty high-affinity and 13 moderate affinity putative NK-2 homeodomain binding sites were found in 2.2 kb of the 5'-flanking region of the *NK-2* gene; hence the possibility of feed-back regulation of *NK-2* gene expression by NK-2 protein was considered. Mutation of the *vnd* gene resulted in large decreases

in the number of neuroectodermal cells and neuroblasts that express the *NK-2* (i.e., *vnd*) gene and also reduced the levels of NK-2 mRNA in cells. These results suggest that NK-2 protein may be required for maintenance of *NK-2* gene expression. A project was initiated to identify nucleotide sequences in the 5'-upstream region of the *NK-2* gene and DNA binding proteins that regulate *NK-2* gene expression. The 2.2 and 8.4 kb DNA fragments from the 5'-upstream region of the *NK-2* gene were subcloned in a vector that contains an enhancerless chloramphenicol acetyltransferase (CAT) reporter gene. *Drosophila* Schneider S2 cells were co-transfected with NK-2 DNA-CAT vector constructs and a vector containing constitutively expressed NK-2 cDNA. Insertion of the 2.2 kb NK-2 DNA fragment in an enhancerless CAT vector resulted in a 5-fold activation of CAT gene expression in transfected Schneider cells due to an endogenous activator. Co-transfection of Schneider cells with a 2.2 kb NK-2-DNA-CAT vector construct and a vector containing constitutively expressed NK-2 cDNA markedly inhibited CAT gene expression. Activation of the NK-2-DNA-CAT reporter gene by constitutively expressed dorsal cDNA also was inhibited markedly by NK-2 protein. These results suggest that NK-2 protein can repress *NK-2* gene expression. The possibility that both NK-2 protein and additional species of protein together may activate the *NK-2* gene deserves further study.

DNA encoding a 77 amino acid residue protein consisting of the NK-2 homeodomain and flanking amino acid residues was expressed in *E. coli* and purified. Additional results on the 3-dimensional structure of the NK-2 homeodomain were obtained by nuclear magnetic resonance spectroscopy. Previous results showed that binding of the NK-2 homeodomain protein to a high-affinity NK-2 binding site in DNA results in an increase in the length of the DNA recognition α-helix III of the NK-2 homeodomain from 11 to 19 amino acid residues. Interaction of the NK-2 homeodomain with DNA stabilizes the conformation of the homeodomain protein.

The primary specificity determinants of high affinity NK-2 homeodomain binding to DNA were shown to be interactions between Ile-47 of the NK-2 homeodomain and A3 and A4 residues in the (+) strand of DNA and between Tyr-54 and T4, C5, and A6 in the (-) strand of DNA. Mutagenesis of DNA encoding the homeodomain of NK-2, which resulted in the replacement of the codon for Tyr-54 by a codon for Met-54, reduced the affinity of the mutated NK-2 homeodomain for the consensus NK-2 binding site in DNA by about 10-fold; whereas, replacement of His-52 by Arg-52 or replacement of Thr-56 by Trp-56 had little effect on the affinity of mutant NK-2 homeodomains for DNA.

Transgenic Lines of *Drosophila*. Previously transgenic lines of Drosophila were obtained by transposition of a P-element that contains a β-galactosidase reporter gene and fly lines were obtained that express β-galactosidase in embryos only in the nervous system. Genomic DNAs adjacent to the P-element insertion sites and cDNAs were cloned from 5 transgenic fly lines.

1. The P-element in transgenic *Drosophila* line 367 was shown to be inserted in a novel zinc finger gene that is expressed

primarily in the CNS of embryos. The P-element insertion into chromosomal DNA is a recessive lethal mutation that results in massive morphologic defects in the ventral nerve cord of developing embryos. cDNA corresponding to gene 367 was cloned and sequenced; the encoded protein contains two putative zinc fingers near the C-terminus of the protein.

2. In transgenic fly line 73 a transposed P-element DNA was shown to be inserted in a novel kinesin heavy chain gene (73) that is expressed during embryonic development only in the CNS and PNS. Two overlapping cDNA clones were isolated and sequenced. The length of 73 cDNA is 5095 bp. Kinesin heavy chain protein-73 consists of >1599 amino acid residues. Kinesin-73 protein is closely related to *C. elegans* UNC-104 which is a motor protein that transports synaptic vesicles of neurons to axonal synaptic regions,

3. Transgenic fly line 393C-2 contains a transposed P-element DNA inserted 1.3 kb upstream of a gene that encodes High Mobility Group Protein-D (HMG-D). HMG-D protein is an abundant chromosomal protein of unknown function that contains one DNA binding HMG-domain. HMG-domains have been found in some chromosomal proteins and in DNA binding proteins that bend DNA and/or regulate transcription.

The HMG-D gene is expressed ubiquitiously during early embryonic development but later in embryonic development was found to be exclusively expressed in the central and peripheral nervous systems. HMG-D protein was shown to bind to cruciform DNA with higher affinity than to double-stranded DNA of identical sequence. The homozygous P-element insertion is a lethal mutation. β-Galactosidase staining of 393C-2 embryos revealed many abnormal embryos with twisted, sometimes broken, ventral nerve cords.

4. Nucleotide sequence analysis of the cDNA cloned from transgenic fly line 314 showed that the cDNA corresponds to a novel *Drosophila* gene that was named QM-D. QM-D cDNA encodes a protein that is similar to QM protein, which reportedly binds to Jun and inhibits DNA binding and transcriptional activation by Jun.

5. Transgenic *Drosophila* line 67 contains a transposed P-element that is inserted in a novel gene that is expressed during embryonic development only in small subset of putative CNS neurons. The corresponding cDNA was cloned and sequenced.

Mouse Homeobox and POU-domain Genes. Previously, novel homeobox genes and a novel POU-domain gene were cloned and characterized. Additional homeobox and POU-domain genes, known only from partial sequences, also were cloned and the complete coding regions were sequenced. These studies were continued during the past year.

1. *OG-9*, a novel mouse homeobox gene, was cloned and partially sequenced. Multiple species of OG-9 mRNA were found in 7-17 day old mouse embryos. One species of OG-9 mRNA was found in RNA from adult skeletal muscle; but OG-9 mRNA was not detected in RNA from many other tissues tested. The *OG-9* homeobox gene therefore is expressed by striated muscle cells, or cells closely associated with striated muscle cells.

2. *OG-2*, a novel homeobox gene, was cloned and partially

sequenced. OG-2 mRNA is expressed in 15-17 day mouse embryos, but not in 7 or 11 day embryos. Two major species of OG-2 mRNA were detected in adult mouse striated muscle and trace levels of *OG-2* mRNA were found in cardiac muscle. *OG-2* mRNA was not detected in other tissues tested.

3. *OG-12* is a novel homeobox gene that encodes a protein with a homeodomain that is not closely related to any previously described homeodomain. OG-12 mRNA was found in 7-17 day mouse embryos, and in most adult tissues tested. However, OG-12 mRNA is most abundant in adult mouse skeletal muscle. Two species of OG-12 cDNA, which correspond to alternatively spliced species of OG-12 mRNA were cloned and were sequenced. In addition, 3,455 bp of *OG-12* genomic DNA were sequenced.

4. *OG-35* is a novel mouse homeobox gene that was cloned and partially sequenced. *OG-35* encodes a protein with a homeodomain that is 88 percent identical to the homeodomain of *C. elegans unc-4* protein, which determines part of the synaptic circuit of a class of motor neurons. The abundance of OG-35 RNA is maximal in 10-12 day mouse embryos. OG-35 mRNA was detected in RNA from adult mouse brain, spleen, lung, liver, and kidney, and NG108-15 neuroblastoma-glioma hybrid cells.

5. *OG-22* is a novel mouse homeobox gene that is similar to rat *Cart-1* homeobox cDNA. Two species of OG-22 cDNA were cloned that correspond to alternatively spliced species of OG-22 mRNA. *In situ* hybridization with OG-22 mRNA expression was found in the first brachial arch, snout and tongue of 10-12 day mouse embryos. In adult mice, OG-22 mRNA was found in seminiferous epithelium of the testes.

6. A novel mouse homeobox gene, *Nkx-1.2*, was cloned that encodes a protein with a homeodomain that is closely related to the *Drosophila* NK-1 homeodomain. Eight kb of *Nkx-1.2* genomic DNA were sequenced. The deduced amino acid sequence of the Nkx-1.2 homeodomain differs from the *Drosophila* NK-1 and chicken Saxl homeodomains by only 3 and 1 amino acids residues, respectively. Northern analysis of poly-A^+ RNA from adult mouse tissues revealed one major band of Nkx-1.2 RNA in brain and three bands in testes. Nkx-1.2 mRNA is present in the seminiferous tubules of the testes, with higher abundance in mature spermatozoa. In 12-14 day mouse embryos, Nkx-1.2 RNA was found in the mesencephalon, myelencephalon, spinal cord, vertebrae, and ribs.

7. The patterns of expression of *Brain-1, Brain-2, Brain-4,* and *Scip* POU domain genes were determined in embryonic, postnatal, and adult brain. Each of the four POU domain genes was found to be regulated independently. Expression of *Brain-2, Brain-4, and Scip POU* domain genes is restricted to the nervous system. *Brain-1* is expressed in all regions of the adult brain, but is not restricted to the nervous system. *Brain-2* is strongly expressed by all neuroepithelial cells in the brain.

Neuronal Polarity in Neuromuscular Junction Formation. Rat motor neurons were co-cultured with striated muscle cells. Axons were distinguished from dendrites by immunocytochemical labelling of specific marker proteins (synaptophysin, high molecular weight microtubule-associated protein, and phosphorylated neurofilament

heavy subunit) and by morphological criteria. Axons were found to have at least 10-fold greater ability to induce post-synaptic acetylcholine receptor aggregation on muscle cells then do dendrites. This suggests that 1 or more of the signals for the induction of post-synaptic differentiation has a polarized distribution in the innervating neurons.

The Role of Nerve-Muscle Adhesion in Neuromuscular Junction Formation. Since our previous ultrastructural results indicated close adhesive interactions between the axon and myotube at sites of contact-induced receptor aggregation the possibility is being explored that this type of adhesive interaction is specific to axons as opposed to dendrites by scanning and transmission electron microscopy and immunocytochemical localization of specific cell adhesion molecules. The results indicate that the most intimate physical interactions between nerve and muscle cells are at muscle sites of acetylcholine receptor aggregation induced by contact with axons.

Assembly of the Presynaptic Apparatus. The localization of several presynaptic plasma membrane and synaptic vesicle-associated molecules, including synapsin, synaptophysin, syntaxin, and SNAP-25 was examined in developing co-cultures of motor neurons and striated muscle cells. The results suggest that these proteins become segregated to axons after the first 2 days of co-culture.

Atoptosis Mechanisms in Irradiated Mouse Embryo Forebrain. Gamma irradiation (2-4Gy) of 16-18 day rat embryo forebrain in utero results in nuclear pyknosis and fragmentation in 80 percent of cortical neuroepithelial cells and 50 percent of cortical plate neurons within 5 hours. Fetal brain genomic DNA is fragmented within 3 hours into an oligonucleosomal ladder pattern, which is a hallmark of apoptosis. These morphological changes and DNA fragmentation require ongoing RNA and protein synthesis, as determined by the use of specific inhibitors. These biochemical characteristics confirm previous inferences from microscopy that radiation kills most fetal brain cells by the mechanism of apoptosis rather than necrosis. Irradiation (4 Gy) caused the abundances of most mRNA species, including those of housekeeping genes, to decline by 50-70%. However, irradiation dramatically increased posttranslationally the level of the p53 transcription factor, and this in turn dramatically induced mRNA for the p53-inducible Waf-1/Cip-1 cell-cycle arrestor. Radiation dramatically increased the levels of mRNAs for the c-Fos and JunB "immediate-early" gene products, without increasing the already high levels of mRNA for c-Jun and c-Myc. Irradiation modestly increased the mRNA for Bax, a positive regulator of apoptosis, and decreased the mRNAs for Bcl-2 and Bcl-xL, two negative regulators of apoptosis, as well as mRNA for a protease potentially involved in apoptosis, interleukin-1-beta-converting enzyme. These results indicate that radiation-elicited apoptosis of fetal brain cells is associated with activation of the p53 system, probable increases in transcription factor AP-1 Fos/JunB heterodimers, and an increased ratio of Bax to [Bcl-2 + Bcl-xL] proteins. These inductions may be related to the triggering of apoptosis in these cells.

Regulation of Expression of the HoxA7 Gene. Work has continued on the description of the promoter region of HoxA7 using CAT reporter constructs and expression in NIH 3T3 cells. Deletions from the 5'- and 3'-sides of the promoter region provided further evidence for the presence of positive and negative regulatory elements controlling HoxA7 transcription.

GTP Binding to HPr. The phosphocarrier protein HPr of the E. coli phosphoenolpyruvate:sugar phosphotransferase system binds GTP. A large collection of HPr mutant proteins provided a basis for mapping the topology of interaction of GTP with the protein.

Crystal Structure of HPr from Mycoplasma. X-ray diffraction analysis was used to solve the structure of the protein, which was shown to exhibit an open-faced beta-sandwich topology.

Structure-function Analysis of Enzyme I. Enzyme I from the E. coli phosphoenolpyruvate:sugar phosphotransferase system transfers a phosphoryl group specifically to HPr from E. coli and not to the acceptor proteins from other bacterial species. A recombinant form of the amino terminal domain of Enzyme I exhibits relaxed specificity for phosphoryl transfer to HPr.

Chromosomal Mapping of Sugar Transport Genes of Mycoplasma. The unique arrangement of separate operons for the gene encoding HPr and those encoding Enzymes I and IIA of the phosphoenolpyruvate:sugar phosphotransferase system was further analyzed by chromosomal mapping. The two operons are located at opposite ends of the chromosome.

Phosphate-dependent Regulation of Adenylyl Cyclase and Phosphodiesterase Activities. In intact cells of E. coli, inorganic orthophosphate stimulates the activity of adenylyl cyclase and inhibits the activity of cyclic AMP phosphodiesterase. A model was proposed for phosphate-dependent regulation of cellular cyclic AMP levels by a dual mechanism.

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Section on Enzymes

(1) Nitration of glutamine synthetase. Treatment of unadenylylated glutamine synthetase GS with peroxynitrate results in nitration of about 1.0 tyrosine residues per subunit and oxidation of 5-6 methionine residues per subunit to methionine sulfoxide. This converts the enzyme to a form that mimics the regulatory properties of the adenylylated GS. It is therefore evident that peroxynitrite, which can be formed endogenously by reaction of nitric oxide with superoxide anion, can seriously disrupt the regulatory function of enzymes and membrane receptors, whose activities are controlled by the cyclic phosphorylation and dephosphorylation of tyrosine residues on the proteins.

(2) Increase of Hydrophobicity of Proteins During Aging and Oxidative Stress. The hydrophobicity of proteins extracted from livers of old rats was found to be significantly greater than that of rat liver proteins from young animals. Treatment of rat liver proteins with an alkyl-, peroxy-radical generating system led to an increase in protein hydrophobicity and to aggregation of some proteins, one of which was tentatively identified as glutathione-S-transferase. Thus, the observed age-related changes in structure and activity of some enzymes may be due to oxidative damage provoked by free radical-mediated reactions.

(3) Free Radical-Mediated Changes in Structure and Function of *E. coli* Glutamine Synthetase. Exposure of GS to an alkyl-, peroxy-radical generating system lead to loss of catalytic activity, oxidative modification of tyrosine, histidine, and methionine residues, and conversion to high molecular weight cross-linked aggregates.

(4) Mechanisms of Action of the Thiol-Specific Antioxidant Enzyme. Ability of the thiol-specific antioxidant protein (TSA) to protect enzymes from inactivation by metal-catalyzed oxidation (MCO) systems is partly due to its thiol peroxidase activity that destroys micromolar quantities of hydrogen peroxide generated in MCO systems. TSA can also protect proteins from oxidative damage in the presence by a peroxidase-independent mechanism. Of the two highly conserved cysteine residues in TSA, Cys 47, but not Cys 170, is required for the peroxidase-independent protective activity. Three mammalian proteins homologous to yeast TSA (Mer 5, natural killer enhancer factors A and B) protected GS from inactivation in the absence of high hydrogen peroxide concentrations, but only Mer-5 was able to protect GS from inactivation by the thiol-dependent MCO system.

(5) Reversal of Reactive Oxygen-Mediated Damage to Methionine Residues of Proteins. Treatment of GS with either hydrogen peroxide or peroxynitrite leads preferentially to the conversion of 7 of 15 methionine residues to methionine sulfoxide (MSOX) residues, but had no effect on catalytic activity. In collaboration with R.L. Levine (Section on Protein Function in Disease, LB, NHLBI), it was found that the

oxidized methionine residues are all located on the surface of the enzyme. Because methionine sulfoxide residues can be reduced back to methionine by the action of the ubiquitous methionine sulfoxide reductase, these results underscore the possibility that the introduction of methionine residues on the surface of an enzyme serves as an antioxidant defense function designed to scavenge reactive oxygen radicals and thereby prevent more serious irreversible damage. Results of preliminary studies indicate that the methionine sulfoxide reductase of rat liver declines with animal age, suggesting that the age-related loss of this key enzyme could contribute to the accumulation of altered forms of enzymes during aging.

Section on Intermediary Metabolism and Bioenergetics

A major research interest of this section has to do with the biosynthesis and functions of selenium-containing biomolecules.

For specific insertion of selenocysteine into proteins the esterified form of the amino acid is generated on a special tRNA from an esterified serine. The selenium donor for this process was identified earlier in this laboratory as selenophosphate. Studies on the mechanism of selenophosphate synthesis from ATP and selenide by selenophosphate synthetase suggest that an intermediate step involves the formation of an enzyme-pyrophosphate derivative. Using [γ -^{32}P]ATP the wild type enzyme was labeled in the presence of selenide but a mutant form lacking cysteine-17 which is essential for catalytic activity was not labeled significantly. Upon treatment of the ^{32}P-labeled enzyme with iodine there was significant loss of radioactivity suggesting the presence of a cysteine phosphate intermediate. A bell-shaped pH stability curve also characteristic of a selenophosphate was exhibited by a corresponding amount of the bound ^{32}P. Attempts to isolate ^{32}P-labeled peptides after limited proteolysis were unsuccessful, presumably due to lability of the derivatives. Direct attempts to characterize the putative phospho-enzyme intermediate by ^{31}P NMR failed even though high enzyme levels were used. Current attempts to crystallize selenophosphate synthetase are in progress. Using polyclonal antibodies directed to *Escherichia coli* selenophosphate synthetase, it was shown by immunoblotting that the enzyme is present in numerous rat tissues, calf liver and *Methanococcus vannielii*. This is evidence that selenophosphate, a highly reactive and oxygen labile compound, serves as selenium donor in eukaryotes and archaea as well as in prokaryotes. The *M. vannielii* enzyme was partially purified and shown to differ in amino acid sequence in the N-terminal region from the *E. coli* protein. The overproduced *E. coli* enzyme exhibits a much lower catalytic activity than expected suggesting that a cofactor or the normal substrate is missing. Thus it is important to investigate enzyme from other sources.

A new eukaryotic selenoenzyme was isolated from a human lung adenocarcinoma cell line. The 110 kDa protein consists of two identical 57 kDa subunits. The presence of selenocysteine in the enzyme was established by chemical analysis and FAD was identified as a bound cofactor. The enzyme was identified as an NADPH-dependent thioredoxin reductase. DTNB, 5,5'dithiobis(2-nitrobenzoate),

also is reduced by the enzyme. Since antibodies directed to rat liver thioredoxin reductase which also react with *E. coli* thioredoxin reductase did not cross react with the human lung enzyme the actual catalytic role of the lung adenocarcinoma enzyme is particularly intriguing.

A new selenocysteine-containing protein, about 35-40 kDa, was isolated from ^{75}Se-labeled *M. vannielii* cells. The sequence of amino acid residues 1-62 of this protein differ from protein sequences in the literature.

In continued studies on *E. coli* formate dehydrogenase, an additional purification step was designed that allowed crystallization of the enzyme. The formate reduced enzyme is extremely oxygen labile but under strictly anaerobic conditions in concentrated protein solutions, stabilized with ammonium sulfate and polyethylene glycol 400, crystals of the reduced enzyme were obtained. A procedure for maintaining crystals in glycerol and ammonium sulfate at liquid nitrogen temperature was required for analysis of crystals. Preliminary crystallographic analysis was performed at 2.6 A by Peter Sun, NIAID.

Section on Metabolic Regulation

This section conducts research in four areas: (A) free radicals in biology, (B) mechanistic study of enzyme action and regulation, particularly those involved in cellular regulation via reversible covalent modification of proteins, (C) mechanism of membrane pore formation and resealing induced by changes in membrane potential, and (D) developing methods and theories applicable to biomedical research.

(A) EPR and spin-trapping methods were used to identify and monitor the formation and utilization of free radicals. We investigated (I) the mechanism of advanced glycation end-products (AGEs) formation by studying the reaction between a dicarbonyl compound methylglyoxal and L-alanine. The results revealed that three types of free radicals were generated. They are: a crosslinked radical cation, the methylglyoxal dialkylimine radical cation, the methylglyoxal radical anion, and the superoxide anion radical, generated only in the presence of molecular oxygen. The radical site in the protein crosslinks is stable. It could be a reactive site for the generation of toxic superoxide anions over a long duration and contribute to accelerated oxidative modification of vascular wall lipids. (ii) A possible role of mutated Cu,Zn-SOD found in familial ALS patients. Transgenic mice expressed with SOD(G93A) have been shown to develop ALS symptoms. We have cloned both the wild-type and the mutant cDNA, expressed them in insect cells, purified the expressed proteins, measured their dismutation activities, and determined the active site copper content. Both enzymes contained one copper per subunit and exhibited identical dismutation activity. Thus, the ALS symptoms observed due to SOD(G93A) are not caused by a reduction in SOD activity, but induced by a gain-of-function, likely by the enhancement of the free radical generating activity reported previously by us.

(B) Reversible covalent modification of proteins is a major cellular regulatory mechanism. We found that: (I) Histamine-induced Ca(II) oscillations in HeLa cells is sustained via reversible phosphorylation cycle, which involves the phosphorylation of

IP_3R and the Ca(II) pump by CaMKII and the dephosphorylation by calyculin A inhibitable phosphatases(s). The frequencies regulated either directly or indirectly by Ins(1,3,4,5)P_4, generated by IP3K, a Ca(II)-calmodulin-regulated enzyme. (ii) A Mg(II)-dependent, Ca(II)-inhibitable protein phosphatase (MCPP) isolated from bovine brain has previously been purified and characterized. A partial cDNA sequence of 137 amino acid residues at the C-terminus revealed that MCPP contained a 24-residue segment that is homologous to a region found in 12 other Mg(II)-dependent phosphatase. In addition, the sequence of the last 73 residues of the C-terminal end contained an acidic region, which exhibited significant homology with a human leukemogenic protein termed DEK. DEK when fused to a CAN protein, is a nuclear protein found in a subtype of acute myeloid leukemia. Thus, MCPP may be a nuclear protein phosphatase regulating DEK and SET, another leukemogenic protein serving as a substrate for MCPP. (iii) Nitric oxide can react with superoxide anion to form peroxynitrite, which is capable of nitrating tyrosine in protein. Using synthetic peptides, patented after the tyrosine phosphorylation site of p34cdc2, a protein kinase essential in regulating cell cycles, we showed that this peptide can be nitrated by peroxynitrite and nitrated tyrosine failed to serve as substrate for lck kinase p56lck. These results revealed that nitration of tyrosine will seriously compromise the tyrosine phosphorylation regulatory mechanism, which plays a major role in signal transduction pathways.

(C) Kinetic studies of the electroporated membrane resealing reveal that Ca(II) or a series of non-ionic synthetic surfactants accelerate the process in the low salt medium, but not in the high salt medium, in which resealing is fast.

(D) A strategy of using a virus's own signal sequence to deliver antiviral peptides into cells has been tested. A cell-impermeable peptide inhibitor of HSV ribonucleotide reductase, when conjugated to a signal sequence for the envelope protein of HSV, was able to inhibit the growth of HSV in Vero cells. The carrier peptide exhibits some specificity. For example, this carrier sequence for HSV can enter HeLa cells known to be infected by HSV, but not MOLT-3 cells, which are not infectable by HSV.

Section on Protein Chemistry

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. 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.

A goal of studies on protein folding is to understand the stabilization of complex secondary and tertiary structures in native proteins that leads to their unique conformations. Assembly processes and biological interactions depend on the correct folding of polypeptide chains. If an unfolding reaction can be described as a reversible, two-state transition, the thermodynamics of protein folding/unfolding can

be determined experimentally from measuring the free energy of unfolding as a function of temperature. Differential scanning calorimetry (DSC) directly gives thermodynamic parameters and, in addition, provides some insight into the mechanism of unfolding a biopolymer. Isothermal calorimetry is used to measure stoichiometry and thermodynamic parameters of protein-ligand and protein-protein interactions. Spectral techniques are used to monitor changes in secondary structure and the environment of tryptophanyl and tyrosyl residues (which may be in the same or different protein domains). Ultracentrifugation, HPLC gel-filtration, gel electrophoresis, and light scattering give information on changes in the size and shape of biopolymers.

The enthalpy of dissociation and unfolding of dodecameric Mn•glutamine synthetase from *E. coli* has been determined to be 14 ± 4 cal/g at pH 7 (with an accompanying uptake of ~ 50 protons/mol) by transfers of the protein into urea solutions in an isothermal titration calorimeter. Urea binding increases ~ 9-fold during protein unfolding. Evidence for six sequential two-state transitions during thermal unfolding of the holo-tryptophan synthase multienzyme αββα complex (with bound pyridoxal phosphate) from *S. typhimurium* has been obtained from DSC and spectral measurements at pH 8 with the holo-enzyme complex and the isolated α and ββ subunits. The extent of thermal unfolding for the αββα complex is ~ 70 % and the total heat is 750 ± 25 kcal/mol. The cofactor increases both the stability and linkage between intra- and inter-subunit unfolding domains in tryptophan synthase. Thermally induced unfolding of *Acanthamoeba* myosin II and skeletal muscle myosin have been studied also. Differences in the cooperativity of unfolding reactions of these myosins relate to differences in rod structures and possibly also head-rod interactions. ATP binding stabilizes head regions of both myosins and work is in progress to identify thermodynamic domains. Studies on proton-induced conformational changes of hemagglutinin from influenza virus X31 indicate that the protein adopts a molten globule conformation under fusogenic conditions (*i.e.*, pH 4.9, 37 °C). From pH 7 to 5, a destabilization of tertiary structures and a stabilization of secondary structures in hemagglutinin are observed. Hydrodynamic properties of the ATP-dependent Clp-AP protease of *E. coli* have been determined. In the presence of ATPγS, ClpA associates to a hexamer which can form enzymatically active 1:1 (21*S*) and 2:1 (28*S*) complexes with ClpP (a 14-mer).

Section on Protein Function in Disease

Research in this section focusses on oxidative modification of proteins, a covalent modification which has been implicated in important physiologic and pathologic processes. These include the aging processes, arthritis, atherosclerosis, AIDS, gene expression, hypertension, intracellular protein turnover, oxygen toxicity, and reperfusion injury after ischemia. Research goals include determination of the chemical and structural changes induced by oxidation; identification of the processes affected by oxidative modification; purification and characterization of the systems which catalyze the modification and subsequent repair or proteolysis; understanding the controls which regulate the modification and disposition of specific proteins; and

application of this knowledge to the rational design of agents which affect these processes.

In the last year, special emphasis was placed in three areas: (1) study of the most highly oxidatively modified protein in rat liver; (2) glutathiolation as a reversible covalent modification of proteins; and (3) detection and quantitation of oxidatively modified proteins.

Several laboratories have established an increased total burden of oxidatively modified proteins during the aging process. Utilizing electrophoresis and isoelectric focussing we noted the presence of a highly oxidatively modified protein in liver extracts from young, male rats. The amount of protein decreases during aging, although the extent of oxidative modification does not change. The protein was identified as carbonic anhydrase, isozyme III. We purified the enzyme from 2, 10, and 18 month old rats to determine changes associated with aging. The enzyme has three known catalytic activities. The specific activities for carbon dioxide hydration and for ester hydrolysis decreased during aging by a modest 30%. However, the third activity, that of a phosphatase, was virtually lost during aging. Studies with small substrates and peptides established that the activity is that of a tyrosine-phosphate phosphatase, thus implicating carbonic anhydrase III as a participant in cell signaling.

Reversible covalent modification of proteins is a fundamental mechanism of cellular regulation. We have found two examples in which glutathiolation of cysteine residues reversibly affects enzyme activity. The first is carbonic anhydrase III, which is known to undergo increased glutathiolation *in vivo* in response to oxidative stress. The loss of phosphatase activity during aging is associated with a doubling of glutathiolation, suggesting that glutathiolation caused the loss of phosphatase activity. Experimental investigation showed that the opposite actually is the case; glutathiolation of Cys_{188} is required for phosphatase activity. The second example is that of the protease from HIV-1, an enzyme essential for viral replication. The protein has two conserved cysteine residues, including the surface-exposed Cys_{67} which had previously been considered unimportant for catalytic activity. However, glutathiolation of that cysteine tripled the activity of the protease. Moreover, glutathiolation virtually eliminated auto-proteolytic degradation of the enzyme. Thus, glutathiolation of the HIV protease both activates and stabilizes the enzyme. We propose that glutathiolation may be an important regulation of cellular metabolism *in vivo*.

Assessment of oxidative modification was advanced by the development of two methods. The first allows for detection and quantitation of 2-oxo-histidine during amino acid analysis, providing a specific marker for oxidative modification. The second is a sensitive and rapid technique to locate covalent modifications in proteins by simultaneous sequencing of peptide collections. It was successful in identifying (1) oxo-histidine as the oxidation product of histidine in glutamine synthetase; (2) the site of glutathiolation of carbonic anhydrase; and (3) the sites of methionine sulfoxide formation in proteins exposed to oxidizing conditions.

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The areas currently under study and development by the five senior members of the Laboratory include nuclear magnetic resonance and computer modeling of proteins and protein expression (Ferretti), small molecule high resolution x-ray crystallography, scanning tunnelling and force field microscopy (Silverton), countercurrent chromatography (Ito), biocalorimetry and near infrared spectroscopy (Berger), peptide, protein, and oligosaccharide mass spectrometry (Fales).

Ferretti continues his studies on peptides and proteins in the free and bound states in an effort to understand structure-activity relationships. Solution and solid state nuclear magnetic resonance (NMR) spectroscopy are being used along with computer modeling (minimum energy computations, simulated annealing, and restrained molecular dynamics) to calculate three dimensional structure. Ancillary techniques are circular dichroism spectropolarimetry, mass spectrometry, and low angle neutron scattering. To obtain sufficient protein for these studies, he is currently using standard protein expression techniques in minimal media to obtain uniformly isotopically labelled materials.

This year he has further refined the three dimensional structure of the 77 amino acid homeodomain protein NK-2 from *Drosophila melanogaster* (synthesized in *E. coli*) bound to its consensus DNA binding sequence. Homeodomain proteins are conserved throughout evolution and represent the DNA binding region of homeotic genes, which control development. As mentioned in the last report, in the absence of DNA three helical segments including a helix-turn-helix domain were identified. The third helix, the DNA recognition helix, was found to be comparatively short in the absence of DNA. Interestingly, preliminary gel shift and NMR data show that the affinity of the protein for duplex DNA increases when the free protein has a shorter recognition helix; the recognition helix increasing in length from 12 to 19 residues upon binding to DNA. Analysis of the NMR data suggests that mutating three nearby sites in the third helix should alter the recognition and binding constant. These proteins have recently been prepared, and the thermodynamic properties of the protein/DNA recognition and binding are under investigation to elucidate the general rules for homeodomain-DNA interactions. Preliminary CD and NMR experiments have been carried out on these mutations.

The three dimensional structure of partially unfolded states of wild type and mutant cytochrome c proteins ISO2 from *Saccharomyces cerevisiae* is still under investigation, due to its interest as a probe of protein folding. Proton resonance assignments for most of the amino acid residues in the wild type analog have been made and a preliminary model of the unfolding pathway has been developed. A comparison of the spectra of the wild type with three mutants (two single and one double mutant) has permitted him to obtain resonance assignments for the mutants. The global structures of the wild type and the mutants are quite similar although the internal mobilities are significantly different. This difference in internal mobility results from mutation of a single amino acid residue from methionine to leucine, which form part of the hydrophobic core of the protein. This change of one amino acid residue is sufficient to disrupt the stability afforded by the core.

In work at the University of Rome, Ferretti studied the three dimensional solution structure of gellan, a naturally occurring bacterial polysaccharide. There are no published solution structures of complex polysaccharides and it was interesting to discover that gellan forms a double helix whereas certain derivatives form triple helices.

Silverton is interested in determining absolute and relative stereochemistry of compounds having anti-retroviral and enzyme inhibitory activity using x-ray crystallographic. A second activity is the examination of biological structures such as membranes using atomic force microscopy techniques.

This year some aspects of polymorphism affecting the analysis of chiral forms of thalidomide have been studied since, despite its deleterious effects on fetal development, it appears to have promise in AIDS therapy. In related work, several hormone inhibitors, coordination compounds, and the conformation of an unusually large terpenoid compound, secotrinervine, have also been determined. The latter may be of interest in evolutionary studies since they are synthesized by primitive insects which lack the enzyme necessary to convert terpenes to steroids. In these animals, such compounds are used as both pheromones and active defensive secretions.

In the area of atomic force microscopy, Silverton has been involved in developing reliable and reproducible techniques for use of the atomic force microscope under the aqueous conditions appropriate to maintaining the integrity of biological systems. He has successfully solved the otherwise intractable problem of measuring the thickness of membranes typical of cell walls. These membranes are of the order of 25 Angstroms thick and these measurements allow the completion of work by neutron reflection on the complete structure of the membrane.

Ito continues his studies on the separation by pH-zone refining countercurrent chromatography of optical isomers of amino acids by converting them to more lipophilic derivatives. These are separated with the help of chiral selectors added to the stationary phase. Samples sizes up to 2g were separated in 7 hours. The mathematics of the separation process was deduced and agreed well with theory.

In the important area of protein separation, Ito has used CCC to purify recombinant proteins. In extending his recent discovery of pH zone refining countercurrent chromatography, has successfully separated gram quantities of enantiomers of a leucine derivative using a chiral selector in the stationary phase. This technique will undoubtedly be of great interest to the pharmaceutical industry because of their interest in preparing pure enantiomers of chiral drugs. In other work, he has extended the technique to the displacement mode (rather than the usual reverse displacement mode). This offers the advantage of collecting fractions free of salt and the possibility of extending the method to ligand-affinity separations. He has also shown that the method works with bases as well as acids, separating several alkaloid mixtures and gram quantities of di- and tripeptide derivatives.

Berger, working with the Army in their blood substitute program, is concerned with the measurement and characterization of the interactions of allosteric effectors such as 2,3-diphosphoglycerate, protons, chloride ion and carbon dioxide with hemoglobin. Such studies have been hampered in the past by the lack of optical signals associated with their formation. His expertise in measuring thermal effects has been brought to bear on the problem and he has recently constructed oxygen equilibrium apparatus for whole blood and hemoglobin solutions. These instruments will be used along with a very sensitive flow calorimeter of his design to study the binding of endotoxins, etc. on hemoglobins, mutants and modifieds of importance in the artificial blood program.

Fales' emphasis in 1994-1995 was again on examining the conditions under which mass spectra on peptides and proteins can be obtained from the Laboratory's MALDI and electrospray spectrometers. Ion trap development has been temporarily discontinued due to the press of problems presented to the other two instruments. With his technicians, Mr. Sokolosky, who operates the MALDI spectrometer and Mr. Dutky who is in charge of the electrospray spectrometer, Dr. Fales continues to study the processes responsible for development of the multiply charged ions that are crucial to successful protein analysis. This has led to a redesign of the electrospray interface resulting in much improved sensitivity and stability.

With a view to determining the conditions responsible for maximizing charge and understanding cleavage under MS/MS conditions, a series of quaternary polymethylene amines have been prepared and studied under a variety of conditions. An interesting new cleavage reaction of proteins brought about by the use of dry air in the electrospray system appears especially promising. With the

use of the maximum entropy computer routines purchased last year, his group is able to discern sequences at the end and in the middle of certain proteins.

Fales continues his collaborations with over 15 researchers in addition to the daily examination of routine peptide and protein samples done as a service to the many NIH workers who are preparing these compounds by recombinant and synthetic means. In this connection, he sees an important role for mass spectrometry at NIH and has spent much time in instructing others in its applications. He has also trained several researchers sufficiently so that they may use his equipment without his own intervention.

Annual Report of the Laboratory of Cardiac Energetics

National Heart, Lung and Blood Institute
October 1, 1994 through September 30, 1995

The major goal of the Laboratory of Cardiac Energetics is to improve our understanding of the cellular and molecular processes involved in the conversion of energy to useful forms of work in the heart and other tissues. With this insight we hope to develop new strategies for the diagnosis, prevention and treatment of heart disease. Our technological approach to these problems is the use of non-invasive nuclear magnetic resonance (NMR) and optical spectroscopy techniques. These methods permit the non-invasive monitoring of several critical parameters of energy metabolism including metabolites, blood flow and tissue oxygenation in intact tissues or humans. The application of these technologies to humans allows us to evaluate these tools as non-invasive diagnostic modalities.

The major energy metabolism pathway we have been concentrating on over the last year is the complex interaction between energy conversion processes in the heart (i.e. oxidative phosphorylation), muscle contraction (i.e. pumping of blood) and coronary blood flow. For the heart to function properly these three elements must be orchestrated with remarkable accuracy to provide the appropriate amount of oxygen, substrates and energy in the appropriate form to support the pumping of the blood. Myocardial muscle contraction is believed to occur by utilizing the energy in adenosine triphosphate (ATP) produced predominately by oxidative phosphorylation occurring in the mitochondria. To use ATP for muscle contraction, ATP is hydrolysed to adenosine diphosphate (ADP) and inorganic phosphate (Pi). It was long believed that these hydrolysis products of ATP serve as the intracellular signals which regulate the rate of oxidative phosphorylation in the heart. While the further breakdown of ADP to adenosine was believed to regulate the resistance of the coronary blood vessels and thereby control the coronary blood flow. However, as we have previously demonstrated, the hydrolysis products of ATP, ADP and Pi, do not change during physiological increases in work. That is, under conditions where the turnover of ATP has increased almost 5 fold the hydrolysis products of this reaction do not increase. This indicates that the ATP, ADP and Pi levels are highly buffered by oxidative phosphorylation in the healthy myocardium and that these metabolites are unlikely to play an important role in the orchestration of metabolism or coronary blood flow. Hence some other cytosolic parameters must be responsible for the orchestration of these critical processes in the heart. Over the last year we have evaluated new sites where this regulatory processes could be occurring in the control of energy metabolism during work stress.

Another key in the control of energy for oxidative phosphorylation is the mitochondrial NADH/NAD. This redox couple provides most of the energy for oxidative phosphorylation. In the past we have demonstrated that this redox couple also remains constant during increases in workload in vitro. This suggests that the driving force for oxidative phosphorylation is also maintained during increases in work. To further evaluate this process we have developed methods to monitor the NADH/NAD ratio in the intact heart in vivo as well as to evaluate the relation between NADH and the mitochondrial membrane potential and forward activity of the F1-ATPase, the key enzyme in the formation of ATP from ADP and Pi in isolated mitochondria.

We have always tried to use the in vivo condition as the gold standard in the evaluation of metabolic control processes. This is simply due to the fact that once we move to an in vitro preparation the environment becomes very artificial for extended periods of time. For example, in vitro conditions for the heart results in very abnormal flow, oxygen, and carbon substrate delivery properties that result in adaptions which mask normal physiological functions. After working for several years on the perfused heart, we have decided to only use this approach for technical development or added proof with regards to specific mechanisms. Therefore, we have redoubled our efforts in developing optical techniques to monitor extrinsic and intrinsic indicators of physiological and biochemical function in the intact heart in vivo. Over the last year Drs. Andrew Arai and Claudia Kasserra have been working on the development of non-destructive monitoring of NADH, tissue oxygenation, intracellular Ca and extracellular K using various optical spectroscopy techniques. With regard to NADH, a fluorescence method has been developed which apparently corrects or eliminates motion and blood volume artifacts from the exposed porcine heart. This has permitted the monitoring of NADH levels in the intact heart under conditions of ischemia (local occlusion), hyperemia(adenosine), substrate augmentation (ketones) and increased workloads (phenylephrine, dobutamine, afterload etc.). These studies have revealed that the NADH levels do not significantly change with physiological alterations in workload consistent with the in vitro NADH data and in vivo 31P NMR data on high energy phosphates. The fluorescence data also suggested that a net increase in epicardial PO2 is associated with an increase in workload. This was a surprising result since a decrease in oxygen tension was predicted. This latter result was the focus of more studies using reflectance optical spectroscopy.

Using a white light source impinged on the myocardium, the spectral absorbance characteristics of the tissue can be ascertained from the spectral differences of the reflected light. Using this approach we have been able to evaluate the oxygenation status of myoglobin and hemoglobin as well as the redox state of the cytochromes using this approach. In addition, these absorption properties of the tissue are also important in the interpretation of the in vivo fluorescence data since these "inner filters" effect the emission characteristics of the fluorescence light, as we have

previously demonstrated. Using this approach, we have confirmed the notion from the fluorescence data, that the net oxygenation of the tissue is increasing with increasing workload. These results are very important with regard to the regulation of coronary blood flow since it suggests that a decrease in oxygen tension is not required for the hyperemia associated with increases in workload. This is a very similar result to that we have observed in the brain using 1H susceptibility measures during increases in task activation in the motor and visual cortex. Therefore, a feedback between tissue oxygen tension and coronary flow does not seem to be an appropriate model for this process. We have been investigating other models as will be discussed later and in previous annual reports.

The effective clamping of NADH in vivo and in vitro generated interest in the relationship between NADH and the mitochondrial membrane potential which is the primary energy used by the F1-ATPase. Toward this aim, over the last year we have developed an in vitro mitochondria preparation where we can simultaneously monitor NADH fluorescence, respiratory rate (Clark oxygen electrode) and mitochondrial membrane potential. The mitochondrial membrane potential is determined by the distribution of TPP+ using a TPP+ sensitive electrode. Having all of this information required a model to help design appropriate experiments and aid in interpretation of the data. We developed an equivalent circuit model of oxidative phosphorylation where the driving battery for the system was the substrate redox couple and the resistive load on the system was the F1-ATPase activity. Other important sites are the metabolic resistance for NADH formation and the oxidation of NADH by the cytochrome chain. Using this equivalent circuit model and our new data we have been able to determine several key parameters involved in oxidative phosphorylation. These include: the net metabolic resistance for NADH formation for several substrates, the effective redox potential for a given carbon substrate, and the effective forward activity of the F1-ATPase. This latter result is especially important since it permits the determination of F1-ATPase activity in the intact mitochondria. The lack of this assay seriously limited our interpretation of F1-ATPase activities in sub-mitochondrial particle assay of the reverse reaction reported last year. Using this approach we have found a very strong coupling between the NADH levels and the mitochondrial membrane potential. However, when assaying the F1-ATPase activity, by monitoring the rate of ATP formation with a given membrane potential, we found that the forward activity of F1-ATPase was being modulated by nearly a factor of 5 depending on the substrate. By comparing the effective metabolic resistance for NADH formation with the F1-ATPase activity we discovered that the "source" resistance of metabolism was being matched to the "load" resistance of the F1-ATPase. This impedance matching by metabolism results in a maximum power transmission to the mitochondrial membrane potential. How the mitochondria performs this task and how the F1-ATPase is being modulated is actively being investigated. We are currently evaluating the role of intramitochondrial Ca and volume on this process. In any event, these studies reveal that the regulation of oxidative phosphorylation, even at the

mitochondrial level, is much more complex than a simple feedback process and much more work is required to evaluated its control network.

A large portion our of efforts over the last year have been devoted to the development of NMR techniques to the study of organ physiology and biochemical structure in vivo. These approaches are being developed to provide new tools in studying the function of the body in vivo, as well as potentially provide new non-invasive diagnostic tools to clinically evaluate humans.
Using a saturation transfer approach, in combination with standard magnetic resonance imaging (MRI) techniques, we have successfully imaged the rate of magnetization transfer between various macromolecules protons and protons in water in intact tissues. The contrast generated by this process, termed magnetization transfer contrast (MTC), is unique in magnetic resonance imaging and is currently being developed for clinical applications around the world. These applications include the study of multiple sclerosis, blood vessel angiography, AIDS, cancer and sports medicine. This approach has also provided a unique insight into the basic mechanisms of water proton relaxation in biological tissues which we have been concentrating on over the last year.
In our attempts to evaluate the molecular mechanisms responsible for the MTC effect, we have studied a large series of macromolecules and lipids. In summary, these studies have demonstrated that a surface hydroxyl group is the most effective surface group in generating this effect while amine groups can provide a weak effect. Our most recent studies involve the use of specifically targeted deuterium labeling in lipids which effectively isolates different motional domains in the lipid system by preventing a given region to communicate with the rest of the macromolecule due to 2-H inability to exchange magnetization via spin diffusion with 1-H. These studies have revealed that there are different region domains in the lipid bilayer system and that all of these contribute to magnetization transfer in the native lipid. These results also demonstrate that the overall magnetization transfer spectrum is a sum of all of the motional domains in the macromolecule and do not reflect the actual dynamics of the interaction site alone. After characterizing the temperature and field dependence of this process, we became interested in the actual dynamics of water at the interface. Working together with Dr. Simon from Duke University, we were able to measure the self diffusion coefficient of water in oriented lipid samples. This study showed that the translational motion of water is only restricted 3 to 4 fold in these tight biological spaces. This data is critically important in numerous fields concerning the role of water and water dynamics in macromolecular function.

Dr. Wolff has shown using magnetization transfer techniques that low concentration metabolites which exchange protons with water can be detected in intact tissues. This approach amplifies signal in excess of 1000-fold for detecting metabolites such as urea, ammonia and creatine. Dr. Wolff has used this increase in signal to noise to create the first chemical exchange image of the

kidney using this saturation transfer approach. Further studies on the specificity and sensitivity of this measure are underway.

Since November, 1991 the Laboratory has been outfitting a 4 Tesla 1 meter bore NMR system for human studies. This is the highest magnetic field strength available for whole body NMR studies. Only 3 other similar systems are present in the world. This high field has demonstrated that it will improved signal to noise in spectroscopic studies of tissue biochemistry (as much as 3 fold) as well as improve the spatial (3 fold) and time resolution (7 fold) of NMR imaging studies. These advantages have made many new studies possible on humans which could not be performed at the more commonly available fields of 1.5 or 2 Tesla.

In the last year there have been several major technical accomplishments. We have established the contribution of different loss mechanisms associated with making NMR measurements at 170 Mhz in man. Using several coil and phantom(models of human structures) we have established that radiation and dielectric losses can be minimized while the major loss mechanism is the inductive losses induced by both conductive and dielectric currents in the body. Using this information we have developed several new coil designs attempting to limit these loss mechanisms especially the use of radiation shields. Dr. Wen has characterized the dielectric properties of the body at 4T and using a simulation routine established the field dependence of this process. These data indicate that the body may be controlling the deposition of rf energy more than where we place rf coils at 170 MHz. Indeed, a new cardiac and respiratory monitoring system has been developed by Dr. Wen based on these properties. Dr. Wen has also shown that dielectric structures can be used as rf coils with very interesting low loss and high sensitivity properties if the right material is used.

In our studies of the heart, Dr. Wolff and co-workers have developed new magnetic field shimming routines to correct the magnetic field disruptions induced by the chest in the magnet. Using a 3 dimensional chemical shift imaging approach they have been able to map the average magnetic field around the heart with minimal effects of flow. These studies have revealed that significant disruption of the hearts magnetic field can be corrected with up to 3 order. These corrections have resulted in significant improvements in cardiac imaging, especially with fast or frequency selective approaches. Currently, we have begun 3D imaging of human subjects at 1.5 T as well as 4T in collaboration with the Cardiology Branch.

In our human muscle energetic studies Dr. Ryschon have established that there is a negative correlation between blood serum Mg levels and skeletal muscle Mg levels. This latter result is consistent with the skeletal muscle Mg pool being a buffer pool for the serum levels. This hypothesis is currently being considered for further investigation. The muscle energetics studies have also evaluated the differences between concentric and eccentric muscle

eccentric contraction results in a much less energy demand that concentric contraction. Thus, the damage associated with eccentric contraction is not related to the energy state of the tissue but the mechanical disruption by the forced extension. Normalization issues have also been addressed by comparing total muscle volume (from 3D NMR studies), cross sectional area, and maximum voluntary contraction (MVC). These studies are being performed on normals and a variety of clinical conditions to evaluate which parameter or combination will provide the most reliable normalization factor for total muscle mass and power.

Another major project in the LCE is the use of MRI techniques in the evaluation of tissue oxygen levels and blood flow. These studies deal with the development of methods for non-invasive monitoring of perfusion and blood oxygenation, and real-time imaging. The basic experimental tool is the use of Echo-Planar Imaging (EPI), a type of magnetic resonance imaging which acquires images in 0.1 sec. or less, much faster than conventional MRI. The images can be sensitized to spatial variations in blood flow and blood oxygenation, and can be obtained at a rate of up to 1 per second. Other imaging techniques such as Gradient Recalled Echo (GRE), Spiral, and Spin Echo have also been implemented.

In cardiac studies we are utilizing a canine heart model to evaluate the use of GRE to monitor coronary blood flow and tissue oxygenation. Coronary blood flow alters the apparent spin lattice relaxation time (T1) of the tissue water while changes in hemoglobin oxygenation alters the apparent spin-spin relaxation time (T2). Using newly developed segmentation sequences, we have accurately determined the T1 and T2 in the human heart and head at 4T. These studies set the reference points for determining which methods will be most appropriate to determine myocardial blood volume and flow using intrinsic or extrinsic probes. These studies are now being actively pursued.

Annual Report of the Laboratory of Cell Biology
National Heart, Lung, and Blood Institute
October 1, 1994 to September 30, 1995

The Laboratory underwent several organizational changes this year: Dr. Blair Bowers and Dr. Raymond Chen retired, Dr. Theodor Kolobow transferred to the newly formed Pulmonary/Critical Care Medicine Branch, and Dr. Julie Donaldson was recruited into a tenure-track position to begin September 1995. With these changes, the Laboratory contains the following Sections: Cellular Biochemistry and Ultrastructure Section, Edward D. Korn, Chief, H. Brzeska-Bzdega, J. Knutson and Julie Donaldson; Molecular Cell Biology, J. A. Hammer, III, Chief; Cellular Physiology Section, E. Eisenberg, Chief, L. E. Greene; Membrane Enzymology Section, R. W. Hendler, Chief. The Laboratory occupies approximately 9800 square feet in Building 3 and 1500 square feet in Building 10. Major research interests include: (1) the structure and function of non-muscle myosins, (2) the regulation of actin polymerization, (3) the structure and function of the 70-kDa heat shock proteins, (4) bioenergetics, and (5) biological applications of fluorescence spectroscopy. The following summary of major accomplishments in fiscal year 1994 is arranged by scientific topics rather than by organizational Sections.

Acanthamoeba Myosins

Previous work by Dr. Korn and Dr. Hammer described three families of myosin in *Acanthamoeba castellanii*, one myosin II isoform (a classical filamentous myosin with two heavy chains an two pairs of light chains); 3 non-filamentous myosin I isoforms (monomers with a single heavy chain); and a family originally referred to as a high-molecular-weight myosin I and now as myosin V. *Acanthamoeba* myosin II is regulated (inactivated) by a novel phosphorylation of 3 sites at the tip of the tail of each heavy chain while the classical amoeba myosins I are regulated (activated) by phosphorylation of a single amino acid in the globular head of the heavy chain.

This year, Dr. Korn and Dr. Ivan Baines completed and published their quantitative study by immunoelectron microscopy of the localization *in situ* of the active (phosphorylated) forms of myosin IA, IB and IC. It seems reasonable to infer the functions of the three myosin I isoforms from the specific locations of the phosphorylated (active) forms. The results suggest that myosin IA is involved in maintaining cortical tension by generating force between actin filaments in the sub-plasma membrane, actin-rich cortex and in the movement of cytoplasm into pseudopods and phagocytic cups. Plasma-membrane-associated phosphomyosin IB is 10-fold enriched in regions of membrane activity suggesting that it mediates membrane protrusion and invagination by generating force between the membrane and cortical actin filaments. Phosphomyosin IC is specifically associated with actively contracting contractile vacuoles and phosphomyosin IA specifically with small cytoplasmic vesicles.

The myosin I heavy chain kinase that phosphorylates and activates the *Acanthamoeba* myosins I is activated by autophosphorylation which, in turn, is enhanced by association of the kinase with acidic phospholipids and plasma membranes. In addition, kinase

activity is about 50% activated simply by binding to plasma membranes and acidic phospholipids prior to autophosphorylation. This year, Dr. Korn and Dr. Wang demonstrated that autophosphorylation of both soluble and vesicle-bound kinase is predominantly, if not exclusively, intermolecular. More surprising, autophosphorylation of vesicle-bound kinase is intervesicular, i.e. a kinase molecule phosphorylates a kinase molecule bound to another vesicle in preference to one bound to the same vesicle. Similarly, phosphorylation of vesicle-bound myosin I by vesicle-bound kinase appears to be preferentially, if not exclusively, intervesicular even when kinase and myosin molecules are bound to the same vesicles. These *in vitro* results probably have significance for phosphorylation of myosin I by kinase *in situ* where there is both soluble and membrane bound kinase and myosin I.

The regulation of the actin-activated ATPase activity in the head of amoeba myosin II by phosphorylation at the tip of the tail presents an intriguing puzzle. This year, in collaboration with Dr. Rau (NIDDK), Dr. Korn and Dr. Redowicz showed that binding MgATP to the head of dephosphorylated (active) myosin II filaments substantially increased the flexibility of the filament rods (which are about 50-fold more rigid than phosphorylated myosin II filaments whereas MgATP had no effect on the flexibility of phosphorylated (inactive) myosin II filaments. These results provide additional evidence for unexpected communication between head and tail of the helical filaments.

Dictyostelium Myosins

Dictyostelium had been shown previously to contain 5 myosin I genes: myoB, myoC and myoD, are similar to the classic myosin I isoforms originally characterized in *Acanthamoeba*; myoA and myoE are similar but with shorter tails that lack the region corresponding to the second actin-binding site. Drs. Hammer, Wu and Jung have extended their studies of the behavior of single, double and triple "null" mutants and concluded: (i) of the 3 single mutants only myoB$^-$ has impaired motility; (ii) there are only slight synergistic effects on cell motility in double and triple mutants; (iii) only myoB protein is strongly up-regulated during early development; (iv) aggregation-stage cells have much more myoB than myoA or myoC; (v) the role of myoC in supporting efficient cell streaming is seen only in the double mutant; (vi) phagocytosis is impaired in both myoB$^-$ and myoC$^-$ mutants; (vii) double and triple mutants show longer doubling times; (viii) single, double and triple mutants are progressively impaired in pinocytosis; (ix) the efflux of pinocytosed material is impaired in double and triple mutants. Clearly these classic myosins I have overlapping functions in cell motility, phagocytosis and pinocytosis.

Dr. Hammer has more fully characterized another novel *Dictyostelium* myosin, MyoJ, that he initially described last year. The completed sequence of the 258-kDa heavy chain predicts an N-terminal globular S-1-like head, a coiled-coil helix and a C-terminal globular domain. The head has a 30 residue insert and 6 predicted calmodulin sites near the head/tail junction. The predicted overall structure is similar to type V myosins but the sequence of the globular tailpiece is not similar to that of yeast or vertebrate myosins V nor is the head sequence. This myosin may be a new member of the most recently

described new myosin family, myosin XI, which includes 2 myosins from *Arabidopsis* recently sequenced by others.

Melanocyte Myosin V

The myosin V family is one of the better studied groups, other than myosins I and II which have been extensively studied. Mice missing functional myosin V, originally described as *dilute*, appear to be less pigmented apparently because the melanosomes remain highly concentrated within melanocytes ratjer than dispersing throughout heratinocytes. Dr. Hammer and Drs. Wu and Wei have localized myosin V in melanocytes, by immunofluorescence. It stains structures in a punctate pattern in the perinuclear region, at the microtubule organizing enter, and, most intensely, at the tips of dendrites (which also contain actin filaments) where it seems to be associated with end-stage melanosomes. By inference, the punctate staining may represent myosin V passively associated with the shuttle vesicles that carry melanogenic enzymes to the melanosomes, the microtubule-associated staining could be myosin V passively associated with the melanosomes as they move along microtubules to the dendritic tip where myosin V may then be actively involved, together with F-actin, in exocytic events that transfer the melanosomes from the melanocyte to the keratinocyte.

Actin Polymerization

The regulation of actin polymerization through its interaction with one or more of the numerous actin-binding proteins present in all cells has become a central topic of interest in cell biology as it underlies some of the most important aspects of cell structure and function. Two years ago, Dr. Korn and his associates reported preliminary observations showing that muscle F-actin filaments depolymerized rapidly when added to extracts of *Acanthamoeba castellanii* whereas added *Acanthamoeba* F-actin was essentially stable. Other data suggested the presence in the cell extracts of both stabilizing and destabilizing factors that interacted differently with muscle and amoeba actin filaments and there was some indication that either or both factors might be affected by the addition of MgATP. This year, purification of the stabilizing factor suggested that it might be monomeric G-actin. Others had previously shown, and Drs. Korn and Mossakowska have confirmed, that ADP-G-actin has a higher affinity than ATP-G-actin for actophorin, a filament severing protein present in amoeba extracts. Therefore, in the absence of ATP, the high affinity of ADP-G-actin for actophorin might keep the concentration of free actophorin rather low. Addition of ATP could convert the monomeric actin to ATP-G-actin resulting in the release of bound actophorin that could then sever filaments. Further experiments showed that actophorin severs amoeba Ca-F-actin much less efficiently that muscle Ca-F-actin. These experiments, then, illustrate the regulatory effects of nucleotides and divalent cations on the interactions of actin with actin-binding proteins. These results also provide a caution in utilizing readily obtainable muscle actin as a model for the interaction of non-muscle actins with actin-binding proteins. Despite their substantial sequence similarities muscle and non-muscle actins do not always interact identically with actin-binding proteins.

70-kDa Heat Shock Proteins and the Homologous Uncoating ATPase

These studies are directed at understanding the mechanism of action of the 70-kDa class of heat shock proteins. The production of these proteins is greatly increased when cells from bacteria to man are stressed. In addition to their protective role during heat shock, the proteins play a major role as molecular chaperones" in disassembling protein complexes, in facilitating proper protein folding and in protein translocation. One of the defining properties of the hsp70 proteins is their high affinity for ATP and their catalysis of ATP hydrolysis that accompanies their function.

Dr. Greene, Dr. Eisenberg and their collaborators reported last year that a 100-kDa cofactor was required for hsp70 to uncoat reconstructed clathrin baskets *in vitro*. This cofactor protein has now been identified as auxilin, a minor basket assembly protein from brain. In the presence of auxilin, hsp70 can uncoat baskets even in the absence of the hsp70-associated light chains, contrary to previous belief that the light chains were essential for uncoating activity. Auxilin also activates the ATPase activity of hsp70 and can induce binding of hsp70 to baskets in the presence of ATP under conditions where hsp70 will not bind in the absence of auxilin. Thus, hsp70 appears to be involved in the presentation of hsp70 to the baskets.

Previously, the kinetics of uncoating of baskets was observed to have an initial burst followed by a very slow steady state reaction. More recent data show that when the clathrin baskets are free of assembly proteins, there is a fast linear rate of uncoating until the reaction is complete. Thus, it is probably the assembly proteins that prevent hsp70 from undergoing more than one round of uncoating. Hsp70 expressed in *E. coli* has properties similar to that of the native protein. Mutations at residue Asp-10 at he ATP binding site greatly reduce the affinity of the expressed hsp70 for ATP and ADP and its properties in the presence of ATP or ADP resembled those of wild type protein in the absence of nucleotide.

Biological Application of Fluorescence Spectroscopy

Dr. Knutson is attempting to expand the biological applications of time-resolved fluorescence spectroscopy by designing and building new instruments and demonstrating their unique capabilities for the study of macromolecular structures. Technical developments this year include adapting a commercial high pressure spectroscopy cell to his laser system which allowed study of spectra associated with high pressure induced dissociation of tryptophan synthetase and tubulin dimers. New software was developed that allows a better fit of the data to structural models. Stopped-flow instruments are also under design.

Applications of the current instrumentation include studies of protein-DNA binding proteins: transactivators; HIV integrase, interleukin 8 which, contrary to previous reports, was found to remain monomeric under physiologic conditions, immunoglobulin domains, cholera toxin, and the binding of anticancer drugs to tubulin.

Bioenergetics

Energy transduction mediated by proton pumps is a fundamental process both in the conversion by plants of light energy into chemical energy that can either be used immediately by the cell or stored (for example, by photosynthesis) in a form that can be utilized later or in the reverse process in which the stored energy is converted into a form of energy that can be utilized by the cell to support metabolic processes. Dr. Hendler is studying two such systems: cytochrome *c* oxidase (a component of the mitochondrial energy transduction pathway) and bacteriorhodopsin (a component of the light-driven transduction system in the purple membrane of halobacteria.

There are two different bacteriorhodopsin photocycles and the relative contributions of each are regulated by actinic light. Dr. Hendler found that Triton-extraction of specific lipids (specifically, squalene and lipopolysaccharide sulfate) from the purple membrane removes the ability of actinic light to control the process. In fact, exposures of purple membrane to Triton too brief to extract any lipids were sufficient to induce conformational changes in the membrane proteins. The effects of lipid removal could be completely reversed by incubating the Triton-treated membranes with a preparation of sonicated membrane lipids. Utilizing a monitoring system devised in his laboratory, Hendler was able to show that a functional system can be reconstituted by incorporation of bacteriorhodopsin into liposomes

By the use of highly sophisticated instrumentation (a rapid scan, multichannel spectrometer) and data analysis, Hendler and his colleagues concluded that the transfer of 4 electrons from cytochrome a_3 to O_2 involves 4 steps: (1) binding of O_2 to heme a_3, (2) transfer of electrons from heme *a* to a_3, (3) transfer of 1 electron held jointly by heme a_3 and Cu_A to O_2^{2-}, and (4) transfer of the final electron held jointly by heme a_3 and Cu_A to complete the reduction of O_2 to H_2O, These results require revision of several concepts; (1) heme *a* and not, as previously thought, heme a_3 is the first heme to show net oxidation, (2) O_2 is held mostly on Cu_B,d not on heme a_3, during its reduction, (3) heme a_3 is oxidized in two steps, not one, (4) oxyferryl (Fe^{+4}) is not an important intermediate.

SUMMARY REPORT OF THE LABORATORY CHIEF
Laboratory of Cell Signaling, NHLBI
October 1, 1994 to September 30, 1995

I. Signal activated phospholipases like phosphoinositide-specific phospholipase C (PLC) and phosphocholine-specific phospholipase D (PLD) play a crucial role in generating lipid-derived second messengers in response to a variety of extracellular stimuli. This laboratory continues to study the mechanism by which various PLC and PLD isozymes are modulated and furthermore the role of these enzymes in human disease.

1. Decreased expression of PLC-β2 isozyme in human platelets with impaired function

Platelets from a patient with a mild inherited bleeding disorder and abnormal platelet aggregation show reduced generation of IP_3 and mobilization of calcium in response to several agonists, suggesting a possible defect at the level of PLC activation. We have devised a method to fractionate and quantitate the amounts of PLC isozymes in human platelets. With this approach, we showed that relative to normal platelets, platelets from the patient contained ~ one-third the amount of PLC-β2 and 70 to 80 % the amount of PLC-γ2. PLC-β4, the least abundant isoform, was increased fourfold. These results suggest that the impaired platelet function in the patient is probably attributable to a deficiency of PLC-β2 with a contribution from the possible minimal decrease in PLC-γ2.

2. cDNA cloning, splice variants, expression, and purification of PLC-δ4

As part of our continuing efforts to uncover previously unidentified PLC isoforms and gather clues pointing to a regulatory mechanism for δ type PLCs, we screened a rat brain cDNA library using PCR with oligonucleotide primers based on the amino acid sequences conserved in the catalytic domains of δ type PLCs. This effort led us to clone cDNA corresponding to a previously unidentified PLC-δ, which we named PLC-δ4. PLC-δ4 exists in at least two alternatively spliced forms of 90- and 93-kDa. Only the 90-kDa form of PLC-δ4 was visible by immunoblot analysis in most rat tissues, whereas testis contains mainly the 93-kDa form.

3. Tyrosine kinase-independent activation of PLC-γ1 by the concerted action of tau proteins and arachidonic acid

Heat-stable proteins that can activate PLC-γ1 were purified from bovine brains. These proteins were proved to be members of tau, a microtubule-associated family of proteins. The tau proteins are rich in proline and contain two consensus sequences for the binding of PLC-γ1 SH3 domain. The tau-dependent activation required a cofactor, arachidonic acid, and was markedly inhibited by phosphatidylcholine. This suggests

that the hydrolysis of PC and concomitant generation of arachidonic acid by PLA_2 can activate PLC-γ1 at a low concentration of calcium without involving tyrosine kinases. The PLD-dependent hydrolysis of PC can further synergize the activation.

4. Role of PH domain in the regulation of PLC-β isozymes

PLC-β isozymes, which are known to be activated by G-protein βγ subunits, contain a pleckstrin homology (PH) domain. To understand the role of the PH domain in the regulation of PLC isozymes, 16 point mutants including Trp 131, the most conserved residue in all PH domains, were constructed in the PLC-β3 PH domain and expressed in Sf9 insect and *E. coli*. Studies with the W131 mutants suggest that, contrary to suggestions made for other PH-containing proteins, this residue may be not be involved in the interaction with either Gβγ or PIP_2.

5. Purification of PLD and its activator from rat brain

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) and generates phosphatidic acid (PA) and choline. PA has been implicated as a biologically active molecule and can be further metabolized by a specific hydrolase to form diacylglycerol (DAG), a protein kinase activator. Procedures for the purification of PLD from rat brains have been improved using extraction of brain membranes with 1% triton followed by chromatographies on heparin HPLC, Mono S and Mono Q columns. The partially purified rat brain membrane can be activated in the presence of GTPgS by members of the ARF and Rho families as previously reported by others.

Fractionation of rat brain cytosolic proteins on Heparin-Sepharose and DEAE columns yielded multiple activity peaks when assayed for their capacity to activate the partially purified PLD in the presence of GTPgS. Several peaks were proved to contain ARF or Rho A proteins. ARF probably eluted as multiple peaks because it consists of multiple family members. In addition to the activity peaks attributable to ARF and Rho A, we identified another peak with distinct behavior. Further purification of this seemingly novel activator yielded a protein of 21 kDa that does not react with antibodies to ARF, Rho A or Rho B. Activation by this protein was as efficient as that by ARF or Rho family members and required the presence of PIP_2. This suggests that brain PLD activity can be modulated by a variety of small molecular weight G (Smg) proteins.

II. We previously purified a 25-kDa enzyme from *S. cerevisiae* and rat brain that furnishes protection against oxidation systems capable of generating reactive oxygen species like H_2O_2 and $O_2^{\cdot-}$. This protein has recently been identified as a thioredoxin - dependent peroxidase (TPx). At least five different TPx homologs exist in mammalian tissues. But none of them have been associated with known biochemical functions. Four out of the five mammalian homologs, NKEF-A, NKEF-B, MER5, and Orf-06 were expressed in *E. coli* and purified. All of the recombinant proteins provided protection against a thiol-containing metal catalyzed oxidation system, but not against an oxidation system containing ascorbate. Recombinant NKEF-A, NKEF-B, MER5

exhibited peroxidase activity when the reducing equivalents were provided by a thioredoxin system containing thioredoxin, thioredoxin reductase and NADPH but not when the reducing equivalents were provided by a glutaredoxin system containing glutaredoxin, glutathione, glutathione reductase, and NADPH. Orf-06, like NKEF-A, NKEF-B, and MER5, reduces peroxides in the presence of the nonphysiological hydrogen donor dithiothreitol but not in the presence of the thioredoxin system or the glutaredoxin system. Thus, the physiological hydrogen donor for Orf-06 remains to be identified.

Kinetic parameters of NKEF-A, NKEF-B, MER5 were evaluated to assess their catalytic efficiency. MER5 exhibited the highest peroxidase activity (11 µmole/min/mg), followed by NKEF-A (5.6 µmole/min/mg) and NKEF-B (3 µmole/min/mg). The apparent K_m values for H_2O_2 were < 10µM for all three enzymes. The catalytic efficiencies (k_{cat}/K_m) are $>10^7$ $M^{-1}s^{-1}$. Furthermore, immunoblot analysis suggests that these TPx homologs are ubiquitous and abundant proteins in mammalian tissues; e.g., the amount of each isotype ranges from 0.05 to 0.4 % of total cytosolic protein in K-562 human myelogenous leukemia cells. Thus, total catalytic efficiency (k_{cat}/K_mx [E]) of these TPx homologs may exceed that of catalase or glutathione peroxidase in the cytosol of most mammalian cells.

Annual Report
Laboratory of Molecular Cardiology
National Heart, Lung, and Blood Institute
October 1, 1994 through September 30, 1995

The Laboratory of Molecular Cardiology investigates the regulation, expression and function of contractile proteins and homeodomain proteins. We are particularly interested in the mechanisms responsible for regulating the contractile proteins in smooth muscle and nonmuscle cells as well as the factors that govern the expression of the genes encoding the contractile proteins. We focus on vertebrate nonmuscle myosin II, the conventional form of myosin present in all eukaryotic cells. We are also studying other classes of myosin such as myosin I, V and X. In addition, we are studying a particular set of homeobox genes that play a role in the early development of *Drosophila* and mammalian embryos. By studying the genes, mRNA, and proteins involved in developmental and contractile processes, we hope to understand the mechanisms by which cells differentiate, alter their phenotype, migrate, change shape, move membrane receptors, secrete cellular products, and proliferate. We plan to use this information to understand both normal and disease processes.

Below is a summary of the various projects under study during October 1994 to September 1995. The following findings were of particular note: 1) We have used the Baculovirus expression system to produce mg quantities of heavy meromyosin (HMM)-like isoforms of nonmuscle myosin heavy chain-B that, under ordinary circumstances, would be extremely difficult to purify. We have compared the properties of an isoform of myosin heavy chain-B which is only expressed in neuronal cells, to that of the ubiquitously expressed isoform. 2) The *cis*-acting element that plays a major role in regulating the alternative splicing of the mRNA encoding neuronal-specific myosin has been localized to a 20 nucleotide segment. 3) The homeobox box protein, *NK-4*, was found to act both as a transcriptional activator and a repressor and the regulatory elements for *NK-4* gene expression were found to be conserved during evolution. 4) The PRD-repeat domain of the *Drosophila NK-1* homeodomain protein was found to be a protein binding interface.

Much of the work in this laboratory is directed toward understanding the function and regulation of expression of vertebrate nonmuscle myosin. The myosin molecule consists of 2 heavy chains of approximately 200,000 kD each and 2 pairs of light chains of 17 kD and 20 kD. In vertebrates, there appear to be 2 major isoforms of nonmuscle myosin II. These 2 isoforms are defined by their heavy chain and we refer to them as myosin heavy chain-A (MHC-A) and myosin heavy chain-B (MHC-B). The human isoforms are encoded by 2 separate genes located on different chromosomes. In addition, nonmuscle MHC-B can undergo alternative splicing of its pre-mRNA to yield at least three more isoforms in avian and mammalian cells. The locations and the amino acid sequences of these alternatively spliced isoforms are of interest. Similar to other myosin heavy chain IIs, MHC-B can be proteolytically cleaved at two sites, one located about 25 kD and the second about 75 kD from the

amino terminal end. These two proteolytically susceptible sites correspond to regions in the MHC that were not resolved in the 3-D crystallographic structure of chicken skeletal muscle myosin S-1 and probably are present as disordered surface loops. The region at the 25-50 kD junction, termed loop 1, is near the ATP binding domain. The region at the 50-20 kD junction, termed loop 2, is near the actin binding domain. mRNA encoding nonmuscle MHC-B has been shown to generate two different insertions in loop 1, consisting of either 10 or 16 amino acids. Sequence of the human genomic DNA from this area by S. Kawamoto has revealed the presence of two exons, one encoding 10 amino acids and the second encoding 6 amino acids. The 10 amino acid inserted isoform has been shown to be highly expressed in mammalian cerebral cortex and retina (Itoh and Adelstein, *J. Biol. Chem.* 270, 14533, 1995). The expression of these inserted isoforms in nonmuscle cells appears to vary in both a species- and tissue-dependent manner. In *Xenopus*, an almost identical insert of 16 amino acids in MHC II-B is present in all cells examined to date. MHC II-B lacking the insert does not appear to be expressed in *Xenopus*. In contrast to its ubiquitous expression in *Xenopus* cells, the inserted isoform of MHC II-B in avian and mammalian cells is almost always confined to neuronal tissue and neuronal cell lines where it is usually accompanied by expression of the noninserted isoform. Of note, the constitutively expressed *Xenopus* MHC II-B inserted isoform has been shown to be phosphorylated by cyclin-p34^{cdc2} kinase, both *in vitro* and *in situ*, within the inserted region (Kelley et al., *J. Biol. Chem.* **270,** 1395, 1995). In addition to the insert at loop 1, there is a second insert consisting of 21 amino acids that is present in loop 2 of the MHC. This inserted isoform is confined to neuronal cells in both avian and mammalian species.

Growth and Differentiation of Smooth Muscle and Nonmuscle Cells (S. Kawamoto, Z01 HL 01665-20 MC). In an effort to understand the neuron-specific alternative splicing mechanism present in loop 1, Dr. Kawamoto isolated human genomic DNA clones that encode this region of nonmuscle MHC-B. Between exons 5 and 6, which encode part of the ATP binding site and which are spliced constitutively, there are two alternative exons, one of 30 nucleotides and a second one of 18 nucleotides. The 30 nucleotide exon has an unconventional intron donor sequence of GCAAGT. To localize the critical region of mRNA that is required for regulated alternative splicing, Dr. Kawamoto constructed a minigene with deletions and/or mutations. These constructs were transfected into a variety of cultured cells, including Y79 cells, which are capable of inserting the alternative exons into their mRNA. She has identified a 20 nt sequence located approximately 1.5 kb downstream from the 30 nt alternative exon, that is required for neuron-specific splicing.

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction (J.R. Sellers, E.V. Harvey, Z01 HL 01786-16 MC in collaboration with C. Cremo). The major regulatory system responsible for the activation of vertebrate smooth muscle and nonmuscle myosin involves phosphorylation of the 20 kD myosin light chain (MLC). Actin-activation of the myosin ATPase activity requires that these light chains be phosphorylated. This study provides evidence that the neck region of the myosin molecule, in which 6 polypeptide chains (2 MHCs and 2 pairs of MLCs) are in close

proximity, is critical for the regulation of myosin. We have demonstrated that single-headed myosin, prepared by proteolytically removing 1 of the 2 heads, is no longer regulated by phosphorylation, but is constitutively active. Both its actin-activated MgATPase activity and its *in vitro* motility activity were high, even in the absence of phosphorylation (Cremo et al., *J. Biol. Chem.* **270**, 2171, 1995), implying that the 2 heads, or at least 2 regulatory domains (the MLC binding region of the MHC and the 2 MLCs) may be required for regulation.

Neuronal-specific Isoforms of Vertebrate Nonmuscle Myosin Heavy Chains (K. Itoh, R.S. Adelstein, Z01 HL 04208-09 MC). In an effort to understand the function of the 21 amino acid insert found in loop 2 of the MHC, three separate HMM isoforms of nonmuscle MHC-B were overexpressed in COS and HeLa cells. These included MHC-B-HMM lacking the insert, HMM containing the 21 amino acid insert in loop 2, and HMM containing the 10 and 21 amino acid insert in both loop 1 and loop 2, respectively. Immunoblot analysis confirmed expression of these isoforms in both transiently and stably transfected cells. Using the yeast two hybrid screening system, a number of cDNA clones which encoded proteins capable of interacting with the expressed HMM containing the insert at loop 2, were identified. One of these was a previously identified protein called FUS. FUS cDNA was overexpressed in the Baculovirus system and its interaction with the various isoforms of HMM is being studied.

Myosin Phosphorylation and the Regulation of Contractile Activity (C.A. Kelley, R.S. Adelstein, Z01 HL 04210-08 MC in collaboration with I. Baines). The *Xenopus* kidney epithelial cell line (A6) contains both MHC-A and MHC-B, although the amount of MHC-A exceeds that of MHC-B by approximately 100-fold. The purpose of this study was to compare the biological properties as well as the subcellular distribution of these two isoforms. Using isoform-specific peptide antibodies, MHC-A and MHC-B were purified from A6 cells by immunoprecipitation. Using the *in vitro* motility assay, the velocity of movement of actin filaments propelled by MHC-A was four-fold faster than that of MHC-B. The V_{max} of the actin-activated MgATPase activity of MHC-A was about two-fold faster than that of MHC-B. Using immunofluorescent microscopy, MHC-B was found to be present in the cell cortex and diffusely arranged in the cytoplasm. In contrast, MHC-A was notably absent from the cell periphery and was arranged in a fibrillar-type staining pattern in the cytoplasm.

Expression of Mutant Vertebrate Myosin I's (F. Wang, J.R. Sellers, Z01 HL 04213-06 MC). These studies deal with the structure and function of myosin I. The Baculovirus/Sf9 system is being used to express a full-length chicken brush border MHC I along with its calmodulin light chains. The expressed myosin I translocated actin filaments in an *in vitro* motility assay at a rate indistinguishable from that of tissue-purified myosin I. Present studies are using site-directed mutagenesis to study the amino acid residues essential for myosin I activity.

Expression and Function of Human Nonmuscle Myosin Heavy Chain II-B Isoforms (T. Takenaka, R.S. Adelstein, Y. Preston, Z01 HL 04216-05 MC). The purpose of this study is to understand the function of the 21 amino acid insert in loop 2 of MHC II-B. We used the Baculovirus expression system to generate an HMM equivalent of both the noninserted and 21 amino acid inserted isoforms. In addition,

we are expressing an isoform that contains both the 10 amino acid insertion in loop 1 and a 21 amino acid insertion in loop 2.

In Vitro Functional Studies of Human Cardiac Myosin (H. Jiang, F. Wang, J.R. Sellers, Z01 HL 04217-05 MC in collaboration with L. Fananapazir and N. Epstein). Previous work has shown that approximately 20% of patients with hypertrophic cardiomyopathy (HCM) have mutations in their MHC or MLC (Rayment et al., *Proc. Natl. Acad. Sci. USA* **92**, 3864, 1995). In this study, myosin was purified from cardiac biopsies of normal controls and HCM patients. Three different mutations of three different myosin genes, which resulted in single amino acid replacements, were studied. Mutation R719Q in the MHC, M149V in the essential MLC, and D22K in the regulatory MLC, were each compared to normal controls. Both the R719Q and the M149V mutants cause an increase in the rate of actin filament sliding in the *in vitro* motility assay compared to controls. Interestingly, these two residues lie close to each other in the 3-D structure of myosin. In contrast, the D22K mutation showed no effect on the velocity of translocation of actin filaments.

Expression of Nonmuscle Myosin Isoforms in Eukaryotic Cells (M.A. Conti, R.S. Adelstein, Z01 HL 04218-05 MC). In an effort to understand the specific function of MHC-A and MHC-B in vertebrate nonmuscle cells, we have overexpressed fragments of the MHC in both rat basophil leukemic cells (RBL-2H3) and the melanoma cell line, A2058. In RBL-2H3 cells, specific phosphorylation of the myosin rod by protein kinase C has been shown to occur in intact cells stimulated to release their granules. In the melanoma cell line, A2058, which chemotaxes in response to stimulants, myosin is thought to be involved in cell migration. By overexpressing portions of the myosin rod that contain the site for phosphorylation by protein kinase C, but not ATP and actin binding sites, we are attempting to interfere with the process of granule release (RBL-2H3 cells) and with the ability to migrate in response to a chemotactic stimulus (A2058 cells).

Expression and Site-directed Mutagenesis of Nonmuscle Myosin Heavy Chains (R.S. Adelstein, J.R. Sellers, Y.A. Preston, Z01 HL 04219-05 MC). Using the Baculovirus expression system, we can express mg quantities of HMM-like proteins containing two 150 kD MHCs and a pair of the 17 kD and 20 kD MLC. To date, we have produced two isoforms, the MHC-B equivalent of HMM and an isoform that contains the 10 amino acid sequence present in loop 1. A comparison of these isoforms shows little difference in their actin-activated MgATPase activities and their ability to translocate actin filaments in the *in vitro* motility assay. The 10 amino acid insert present in loop 1 can be phosphorylated by a number of proline-directed kinases including cdc2, cdK5, and MAP kinase. Phosphorylation of the single serine present in this 10 amino acid insert does not appreciably alter the actin-activated ATPase activity or its ability to translocate actin filaments in the *in vitro* motility assay.

Function of *Drosophila* NK-homeobox Genes in Mesodermal Cell Differentiation (Y. Kim, Y.M. Lee, R. Bryant, Z01 HL 04221-04 MC). Regulation of *NK-4* gene expression is dependent upon two clusters of E-box sequences at the 5' upstream region of *NK-4*: an E1 cluster for activation by *twist* and an E2 cluster for *NK-4* autoregulation. If E-box clusters are functionally important for the regulation of *NK-4*, then they may also be conserved in other species during evolution. In order to explore this possibility, *D. virilis* genomic DNA libraries were screened with a labeled *NK-4*

homeobox DNA and the *DvNK-4* homolog of *DmNK-4* was cloned. The deduced amino acid sequence of the *DvNK-4* homeodomain was identical to that of *DmNK-4*. Furthermore, an *NK-3* homeobox gene was also found near *NK-4* in *D. virilis* as in *D. melanogaster*. In addition, the E-box clusters were also found to exist in the 5' upstream region of *DvNK-4*. These results indicate that the *NK-4* homeobox gene cluster may play a key role in embryogenesis and that regulatory circuits are conserved during evolution.

Cloning and Characterization of Myosin-related cDNAs from *Xenopus Laevis* (N. Bhatia-Dey, R.S. Adelstein, Z01 HL 04222-04 MC in collaboration with M. Jamrich and M. Taira). The *Xenopus* forkhead gene, *Xfkh3*, encodes a 483 amino acid protein with a highly conserved putative DNA binding domain of 77 amino acids. Using an animal cap induction assay, the forkhead message seems to be induced by activin, but not by bFGF and retinoic acid. Preliminary functional analysis suggests that the mis-expression of synthetic *Xfkh3* mRNA in the animal pole region may sensitize the animal caps to induction by activin. In separate experiments, the mRNA encoding nonmuscle MHC-A in *Xenopus* was found to have two transcripts: a 7.5 kb transcript which is maternal and is expressed at a constant level throughout embryogenesis and an 8.3 kb transcript which is zygotic and is developmentally specific.

Null Mutations of Vertebrate Nonmuscle Myosin Heavy Chains (A.N. Tullio, R.S. Adelstein, Z01 HL 04223-03 MC, in collaboration with D. Accili, NIDDK). The purpose of these studies is to determine whether MHC-B is required for mouse development and function using the technique of homologous recombination. We have disrupted the second exon of the MHC-B gene which contains the initiating methionine and, following transfer of the embryonic stem cells to mice, blastocysts have produced germline inheritance of the disrupted gene. To date, we have analyzed 32 mice, the progeny of heterozygotes containing the disrupted MHC-B gene. One was homozygous for the disrupted gene and, though born, did not survive. The other 31 offspring were either heterozygous or wild-type. Studies of the cause of this apparent embryonic lethality are underway.

Function of *NK-1* Homeobox Gene in Neuro-muscular Synaptogenesis (Y. Kim, S.H. Lee, Y.M. Lee, Z01 HL 04224-03 MC). Previously, we had identified four novel *Drosophila* genes that encode proteins that interact with the *NK-1* protein using the yeast two-hybrid system. We found that all four of the cDNA clones encoded proteins that show a Cys-rich region. In order to investigate which region of the *NK-1* protein could interact with these gene products, various *NK-1* bait constructs were generated and the interaction assays were performed after cotransformation of yeast. In this analysis, we found that the PRD-repeat domain of the *NK-1* protein was sufficient for protein-protein interaction. These results suggest that the PRD-repeat domain functions as a protein binding interface and this interaction may play a role in the mechanism by which the *Drosophila NK-1* homeobox gene functions during its expression in muscle cells and a subset of neuronal cells of the ventral nerve cord and brain.

Regulatory Mechanisms for Nonmuscle Myosin Heavy Chain Gene Expression (N. Beohar, S. Kawamoto, Z01 HL 04225-03 MC). A 2.8 kb fragment from the first intron of the MHC-A gene causes a 3-10-fold increase in transcriptional activity in NIH 3T3 fibroblasts and C2 myoblasts, but not in differentiated C2 myotubes. The

activating portion of this fragment was narrowed down to 100 bp that demonstrated higher activation than that seen when the entire 2.8 kb fragment was used, suggesting the presence of a repressor element. The 100 bp fragment was used as a probe for a gel shift assay using NIH 3T3 fibroblasts and neuroblastoma nuclear extracts. A specific DNA protein interaction was demonstrated.

Interaction of Myosin Binding Proteins with Smooth Muscle and Nonmuscle Myosin (D. Silver, J.R. Sellers, Z01 HL 04226-01 MC in collaboration with H. Ford, S. Zain and V. Shirinsky). This project concerns the interaction of two different proteins with myosin. One of these is KRP (telokin), an independently expressed protein product derived from a gene within the gene for myosin light chain kinase (MLCK). KRP binds with a stoichiometry of 1 mol/mol myosin and an affinity of 7 μM to unphosphorylated smooth muscle and nonmuscle myosin filaments. It prevents the ATP depolymerization of myosin *in vitro* by binding to the S1-S2 junction. The second protein, mts1, belongs to a family of s100 Ca^{2+} binding proteins and is highly expressed in metastatic cells. Both cosedimentation analysis and electron microscopy suggest that mts1 may promote myosin filament disassembly.

Biochemical and Genetic Approaches to the Unconventional Myosins (N. Bonafe, J.R. Sellers, Z01 HL 04227-01 MC). These studies concern the identification and characterization of unconventional myosins. In particular, we are interested in characterizing the kinetics of myosin V MgATPase activity because this myosin has been implicated in membrane trafficking, which might require a different kinetic scheme than that reported for skeletal muscle myosin. We have cloned fragments encoding at least six different unconventional myosins from a *Drosophila* library using PCR amplification.

Ablation of a Neuron-specific Exon from the Mouse Myosin Gene (H. Hwang, R.S. Adelstein, S. Kawamoto, Z01 HL 04228-01 MC). Using the techniques of homologous recombination in mouse embryonic stem cells, we have initiated experiments to ablate the 10 amino acid insert found in loop 1 of MHC-B in mammalian brain tissue. In an effort to understand its function in the brain, we replaced the exon encoding this insertion with the cDNA encoding neomycin. This construct was transfected into embryonic stem cells and, using genomic blot analysis, we were able to identify two clones that have undergone homologous recombination. Preliminary experiments confirm the ability of the altered mRNA to undergo normal splicing.

Annual Report of the Laboratory of Molecular Immunology
National Heart, Lung, and Blood Institute
October 1, 1994 to Sept 30, 1995

The past year has been an extremely successful one in the Laboratory of Molecular Immunology, a new laboratory formed by the merger of the Section on Pulmonary and Molecular Immunology and the Laboratory of Chemical Pharmacology. Research is divided into four sections and five laboratory efforts. These projects areas include three major project areas: (1) Studies focused on an understanding of the T-cell activation process-- studies with importance for immunodeficiency, cancer (including one form of leukemia), and for autoimmune diseases; (2) Studies focused on an understanding of the activation of mast cells-- an area of importance for asthma and other allergic diseases; and (3) Studies focused on the mechanisms of drug-induced toxicities, with particular emphasis on the mechanisms of hepatitis resulting from inhalation anesthetics and nonsteroidal anti-inflammatory drugs. Smaller research efforts on the role of nitric oxide synthase in retina and neurotoxicity in dopamine containing neurons were also pursued.

A. Studies of T cell activation related to the IL-2 receptor-- structure, function, and signaling via IL-2 receptors; relationship to severe combined immunodeficiency disease.

The human interleukin-2 receptor is being studied to understand critical components of the T cell immune response in normal and neoplastic cells. Following T-cell activation by antigen or mitogenic lectin, the magnitude and duration of the T-cell immune response is determined by the amount of IL-2 produced, the levels of receptors expressed, and the time course of each of these events. Three IL-2 receptor chains are now known to exist: IL-2Rα, IL-2Rβ, and the common cytokine receptor γ chain, γ_c. Other proteins may additionally contribute to a multichain receptor complex. In the past year, major advances have been made related to our understanding of inherited human immunodeficiency and cytokine signaling.

1. Mutations in γ_c as the cause of XSCID and elucidation that the γ_c chain is a component of the IL-2, IL-4, IL-7, and IL-9 receptors but not the IL-13 receptor. Previously, it was shown that mutation of γ_c results in X-linked severe combined immunodeficiency (XSCID) in humans. Moreover, γ_c was shown to be a component of the IL-4 and IL-7 receptors, a finding that clarified why the phenotype in XSCID was more severe than in humans or mice with IL-2 deficiency. Now, the Section of Lymphocyte Activation has additionally demonstrated that γ_c is also a component of the IL-9 receptor. Published data indicated that IL-4 and IL-13 receptors shared a common component. With the discovery that γ_c was shared by the IL-4 receptor, it was assumed that γ_c was also a component of the IL-13 receptor, but this has been disproved. The group has now demonstrated that there are two types of IL-4 receptors, one that contain γ_c and one that does not. The second type of IL-4 receptor is believed to be formed by a combination of IL-4R and IL-13R, and should be capable of responding to both IL-4 and IL-13. Efforts to clone the still elusive IL-13R are planned.

2. Less severe forms of immunodeficiency can be caused by γ_c mutations. The group reported that γ_c is also the genetic defect in at least one form of moderate X-linked combined immunodeficiency (XCID).

3. Association of IL-2Rβ and γ_c with Jak1 and Jak3, respectively; SCID associated with Jak3 deficiency. Based on previous observations that heterodimerization of IL-2Rβ and γ_c is required for signaling, the group investigated and then in the past year reported that IL-2Rβ and γ_c associate with two different Janus family tyrosine kinases (Jak1 and Jak3, respectively). Interestingly, the patient with XCID (above) has a single amino acid change in the γ_c cytoplasmic domain that decreased association with Jak3. This led to the hypothesis that Jak3 activation is essential for intrathymic maturation and/or selection of T cells and that XSCID results from γ_c mutations that interfere with cytokine binding to γ_c and/or the ability of γ_c to associate with Jak3. This hypothesis led to the prediction that Jak3 would be found in some autosomal recessive cases of immunodeficiency that are phenotypically similar to XSCID or XCID. Indeed, such a case of autosomal recessive SCID due to defective Jak3 expression has been found.

4. Studies of IL-2 signaling. The group developed and used a powerful system for studying IL-2 signaling, namely the transfection of 32D myeloid progenitor cells with wild type or mutant IL-2Rβ constructs. 32D cells are normally dependent on IL-3, but after transfection with IL-2Rβ, they can also respond to IL-2. 32D-β cells are valuable for

studying IL-2 signal transduction since these cells respond to IL-2 but do not require IL-2 for survival. Tyrosine kinase inhibitors such as herbimycin A inhibited both IL-2 induced proliferation and IL-2 induced IL-2Rα mRNA expression in 32D-β cells, indicating that tyrosine kinase(s) are required for IL-2 induced signaling, and these cells were used to demonstrate that IL-2 regulated apoptosis and bcl-2 expression in these growth factor dependent cells. By transfecting these cells with IL-2Rβ constructs in which the tyrosines were individually or collectively mutated, it has been possible to correlate the proliferative response to IL-2 with the requirement of specific cellular pathways. This approach has proved valuable in analyzing proliferation versus differentiation. The group also had used the 32D system and chimeric receptor constructs to demonstrate that heterodimerization of IL-2Rβ and γ_c is required for IL-2 signaling: if cells were transfected with constructs in which the extracellular domains of IL-2Rβ and γ_c were replaced with that from IL-2Rα, then antibodies to IL-2Rα could trigger proliferation when mixtures of the α/β and α/γ chimeric constructs were used but not when either was expressed by itself. The approach was extended using specific mutants of the cytoplasmic domain of γ_c in order to clarify the residues/regions required for signaling.

5. Role of γ_c in lymphoid development: preparation of $_c\gamma$ deficient mice. $_c\gamma$ deficient mice exhibit the following properties: (a) Small hypoplastic thymuses that nevertheless retain all populations of thymocytes, although the CD4:CD8 ratio is increased. (b) Small hypoplastic spleens at 3 weeks of age that increase in size in an age related fashion by 4 weeks of age, with a progressive increase in CD4+ T cells. (c) No NK cells. (d) No dendritic epidermal T cells but normal Langerhans cells. (e) No γδ intestinal intraepithelial cells. (f) No gut associated lymphoid tissue. (g) Greatly diminished conventional B cells in bone marrow and spleen, yet peritoneal B1 cells were readily detected. (h) Diminished IgG, IgA, and IgE, but normal levels of IgM. (i) Diminished proliferation to γ_c-dependent signals, but striking proliferation of thymocytes PMA + ionomycin and to anti-CD3 + anti-CD28. (j) Greatly diminished IFN-γ production; more modest decrease in IL-2 and IL-4 production. (k) Inflammation of the cecum and colon, possibly correlating with Helicobacter hepaticus colonization. A striking finding in the mice was that although T cell numbers were greatly diminished, T cells were produced and could progress to the single positive phase; in addition potent thymocyte proliferation could be induced by anti-CD3 + anti-CD28 or PMA + ionomycin. This suggests that either cytokine independent mechanism(s) or γ_c-independent cytokine(s) mediated events previously assumed to be strictly dependent on γ_c. It was also striking that although human XSCID patients have normal numbers of B cells, γ_c-deficient mice had greatly diminished conventional B cells. A likely explanation is that inactivation of IL-7, a potent pre-B cell growth factor in mice, prevents B cell development in mice, whereas in humans, IL-7 is less important as a pre-B cell growth factor. This hypothesis is supported by the phenotype of IL-7 and IL-7 receptor knockout mice. The mice are now being reconstituted by transgenic constructs in which γ_c expression is driven in a lineage-specific fashion.

6. Gene therapy for XSCID. Adeno-associated viral and retroviral constructs have been made. The group has now reported the successful transduction of fibroblasts using an amphotropic γ_c retrovirus. In addition, bone marrow and cord blood cells have been "tagged" successfully by neomycin resistance. Now that the group has successfully prepared γ_c-deficient mice as an animal model for XSCID, these will be used as initial animal targets for gene therapeutic retroviruses. If results are successful, this will facilitate the eventual trials oriented towards human gene therapy.

7. Range of expression of γ_c. In addition to expression on T and B cells, it is now clear that monocytes and neutrophils express γ_c. Interestingly, γ_c is inducible on monocytes (at both the protein and mRNA levels) in response to IL-2 and γ-interferon.

8. Identification of γ_c associated molecules. The yeast two-hybrid system has been used in an effort to identify molecules that associate with the cytoplasmic domain of γ_c. Results are encouraging and candidate proteins identified by this method are being studied.

B. Regulation of T-cell activation-- regulation of IL-2 receptor gene expression and studies of cytokine induced STAT proteins

This laboratory was the first to analyze the promoters of each of the three chains of the IL-2 receptor, namely IL-2Rα, IL-2Rβ, and γ_c. IL-2Rα and IL-2Rβ are significantly regulated at the level of transcription and their expression is increased in response to both mitogens and IL-2. Major advances have been made related to the study of the IL-2R gene and in characterizing the STAT proteins (signal transducers and activators of transcription) induced by IL-2, IL-4, IL-7, IL-13, and IL-15. These are latent transcription factors that couple signaling from the cell surface

to the nucleus.

1. IL-2Rα chain gene regulation: The IL-2Rα chain 5' regulatory region was previously shown to contain an important element, denoted positive regulatory region I (PRRI) that spans an NF-κB site, a CArG motif, an Sp1 site, and a fourth site denoted NF-IL-2RA, all located between -299 and -228 relative to the major transcription initiation sites. We have now reported the existence of a second essential element, PRRII, more proximal to the transcription initiation site. This element contains binding sites for at least two transcription factors-- Elf-1 (an Ets family protein) and HMG-I(Y) (11-12 kDa high mobility group proteins that are highly expressed in rapidly proliferating cells). Elf-1 is critical for IL-2Rα promoter activity; a single nucleotide change within the Elf-1 site is sufficient to abrogate IL-2Rα promoter activity. Elf-1 physically associates with HMG-I(Y), NF-κB p50, and c-Rel in vitro. This exciting finding provides a logical basis for functional coordination between PRRI and PRRII. Moreover, Elf-1 also physically associates with TFIIB, providing a basis for interaction with the basal transcriptional machinery. HMG-I(Y) is a minor groove binding protein that most likely serves a framework function, perhaps serving to bend DNA. Further upstream, a γ-interferon activated sequence (GAS) motif has been identified that likely plays a role in IL-2 induced regulation of this gene. This possibility is being examined.

Interestingly, a binding site selection analysis has revealed that the consensus motif for Elf-1 is not found in any known Elf-1 regulated genes. Instead, Elf-1 binding appears to be dependent on the presence of accessory proteins that facilitate its binding to lower affinity sites. Mutagenesis of low affinity Elf-1 sites in the context of the IL-2Rα promoter or the GM-CSF enhancer results in a lower level of inducible activation. This suggests that low affinity sites are favored as a mechanism of allowing more potent transcriptional activation. Moreover, another Ets family protein (denoted ERGB/Fli1) can bind to the optimal Elf-1 site, but not for example to the naturally occurring low affinity IL-2Rα site. As a result, it is possible that high affinity sites, if they occurred, would be too nonspecific and that other Ets family proteins might also bind. Thus, the low affinity binding sites may allow not only for greater inducibility but also for greater specificity of gene expression.

2. Cytokine induced STAT proteins. Considerable energy has been devoted to the study of STAT proteins induced by cytokines whose receptors contain γ_c. These latent transcription factors are rapidly induced by tyrosine phosphorylation. We have in fact evaluated the abilities of IL-2, IL-4, IL-7, and IL-15 (whose receptors contain γ_c) as well as IL-13 (whose receptor does not contain γ_c) and have found that all induce STAT proteins. Interestingly, IL-2, IL-7, and IL-15 all induce Stat5 in freshly isolated peripheral blood lymphocytes (PBL) and both Stat3 and Stat5 in PBL preactivated for 72 hours with PHA. In contrast, both IL-4 and IL-13 induce Stat6. We evaluated the abilities of phosphorylated peptides spanning the tyrosines contained in IL-2Rβ and γ_c to inhibit Stat binding activity and in this fashion we implicated Y392 and Y510 of IL-2Rβ as the putative docking sites for Stat proteins. γ_c appears not to play a role in the docking of Stat proteins. These findings were confirmed by making stable transfectants with mutated forms of IL-2Rβ. Strikingly, the IL-7R contained a motif similar to those found at Y392 and Y510 of IL-2Rβ. Thus, it appears that IL-2, IL-7, and IL-15 activate the same Stat proteins because IL-2Rβ and IL-7R contain docking sites for the same Stat proteins. The IL-4 and IL-13 receptors both contain the IL-4R, which is capable of serving as a docking site for Stat6. Thus, the basis for specificity of Stat protein activation is contained within the cytoplasmic domain of the respective receptor molecules.

3. Constitutive versus inducible Stat protein activation associated with HTLV-I transformation. Following infection of T cells by human T-cell lymphotropic virus type I (HTLV-I, the cause of adult T cell leukemia and tropical spastic paraparesis), there is an initial period of IL-2 dependent growth. Over time, by an unknown mechanism, the growth becomes independent of IL-2. Interestingly, although IL-2 is no longer produced in this phase, IL-2 receptor expression is extremely high. In examining STAT protein activation in HTLV-I transformed cells, we found that in growth factor independent established cell lines such as HUT-102B2 or MT-2 cells, STAT proteins are constitutively activated. Following infection of cord blood cells with HTLV-I, STAT proteins are still induced in normal fashion in the early IL-2 dependent phase, but with additional time in culture and the acquisition of IL-2 independence, STAT proteins were constitutively activated. The STAT proteins activated were Stat3 and Stat5, the STAT proteins induced by IL-2. Although the mechanism of STAT protein activation is unknown, neither HTLV-I encoded Tax nor p12 proteins appear to be sufficient for this process. It is striking that the IL-2 receptor complex is constitutively assembled in HTLV-I infected cells. It has been possible to coprecipitate Jak3 with Stat5 as well as with IL-2 receptor chains without adding exogenous IL-2. The constitutive activation of the Jak-STAT pathway suggests that this pathway may play a role in

HTLV-I mediated leukemogenesis.

4. Cloning of human Stat5. Since Stat5 is the principal STAT protein induced by IL-2, we cloned human Stat5 (originally Stat5 was cloned from sheep as a prolactin-induced mammary gland factor, MGF, and later from mouse). cDNAs encoding two closely related proteins, greater than 90% identical at the amino acid level, were identified. Both genes are located on chromosome 17. Two forms have also been identified in the murine system. Experiments in which each Stat5 cDNA was expressed in conjunction with Jak1, Jak3, IL-2Rβ, and γ_c have indicated that each Stat5 form can be individually activated and mediate binding to DNA. The roles of Jak1 and Jak3 in the activation of these proteins is being evaluated. Both forms of Stat5 appear to be identically expressed: mRNA for each is found in T cells, NK cells, and B cells. So far, the data are consistent with the two proteins serving redundant fashions; however, it is possible that unique specificities will be found to result from homodimers of each protein and from heterodimers of the two. An alternatively spliced form of one of the cDNAs has been found. This form is predicted to encode a Stat5 in which the DNA binding domain is deleted. Although it is attractive to hypothesize that such a form would be physiologically regulated to generate a dominant negative, this form is expressed at extremely low levels in normal lymphocytes, either at rest or following activation with PHA.

C. Activation of Mast Cells

Tissue mast cells respond to antigens, via receptors for IgE (FcγR1), with rapid discharge of secretory granules and synthesis of arachidonic acid-derived metabolites and, after a delay, synthesis and release of various cytokines to initiate both immediate and delayed inflammatory reactions. Previously, the major signaling components were identified for each of these functional responses using RBL-2H3 cells, which like mast cells, contain FcγR1 and a novel adenosine A_3 receptor that serves to synergize secretory responses to antigen and other mast cell secretagogues. In addition, cells that express muscarinic m1 receptors have established. Interestingly, whereas the A_3 and m1 receptors recruit G proteins, FcγR1 recruits the tyrosine kinases Lyn and Syk. Nevertheless, all three classes of receptor mediate activation of phospholipases (PL) C and D, mobilization of intracellular and extracellular Ca^{2+} and the activation of protein kinase C (PKC), Ca^{2+}-dependent myosin light-chain kinase, and mitogen-activated protein (MAP) kinases. One distinction, however, is that stimulation via the A_3 receptor elicits a transient activation of PLC and MAP kinases but sustained activation of PLD and PKC. Studies this year established that the sustained activations of PLD and PKC provide the primary synergizing signal for secretion. Thus, release of secretory granules is dependent primarily on elevation of Ca^{2+} activation of of certain isoenzymes of PKC through generation of diglycerides via PLD or PLD.

It was also demonstrated that generation of arachidonic acid required the activation of a cytosolic PLA_2 through phosphorylation by p42 MAP kinase ($p42^{mapk}$) and a Ca^{2+}-induced translocation of this enzyme to membranes. The process is inhibited at an early step of the MAP kinase/PLA_2 cascade by nanomolar concentrations of dexamethasone. Inhibition likely occurs at the level of Ras with Raf because events preceding this interaction are unaffected whereas phosphorylation of Raf and subsequent events are inhibited. Moreover, the entire MAP kinase/PLA_2 cascade is activated by elevation of free Ca^{2+}, activation of PKC or engagement of trimeric G proteins. Each of these pathways can be selectively inhibited or activated. The ability of these pathways to activate the MAP kinase/PLA_2 cascade is blocked by dexamethasone or, in permeabilized cells, with GDPγS. These and other studies indicate discrete pathways for activation of the cascade that converge at Ras/Raf. Genetic approaches are being used to define the locus of action of dexamethasone and to determine whether the effects noted above account for its immunosuppressive actions.

Regarding cytokines, production of TNFα requires activation of PKC, elevation of free Ca^{2+} and probably a third signal, either the activation of MAP kinase or recruitment of a tyrosine kinase. Genetic studies will allow evaluation of the role of MAP kinase in this process. Interestingly, release of newly formed TNFα occurs via the Golgi and is totally dependent on PKC and increases in free Ca^{2+}. Thus, release of preformed secretory granules and Golgi-derived vesicles appear to be regulated by the same signals. In contrast to this scenario, TGFβ, a cytokine with anti-inflammatory properties, is constitutively produced and secreted via the Golgi in a fashion not influenced by cell stimulation.

Efforts are now increasingly directed towards the mechanisms targeted by these pathways and potential sites for therapeutic intervention. Since RBL-2H3 cells lack myosin heavy chain (MHC)-B and express exclusively MHC-A, they offer a unique model for studies of the role of MHC-A in exocytosis and other cellular responses to antigen.

Interestingly, MHC-A is phosphorylated in response to antigen in these cells. The classic mast cell secretagogue, compound 48/80, was previously shown to act via direct activation of $G_{\alpha i\text{-}3}$ in peritoneal mast cells, yet it does not act on RBL-2H3 cells. However, following prolonged exposure to quercetin, a MAP kinase cascade suppressant, RBL-2H3 cells can respond to 48/80 and neuropeptide secretagogues. This transition is accompanied by changes in content and localization of in $G_{\alpha i\text{-}3}$ particulate-type structures. These findings provide a provocative basis for further studies of the role of G proteins in trafficking of secretrory granules and vesicles.

D. Mechanisms of pathologies caused by reactive metabolites of drugs and endogenous molecules

Although many life-threatening drug reactions are reported each year, very little is known about their etiologies. Accumulating data now indicate that many of these toxicities as well as other pathological conditions might be caused by the covalent alterations of specific macromolecules by reactive metabolites. These modifications might lead directly to toxicity by changing the physiological functions of macromolecules or indirectly by making macromolecules immunogenic, leading to immune-mediated toxicities. The approach taken by the Section on Molecular and Cellular Toxicology has been to identify the macromolecular targets of reactive metabolites of drugs and endogenous molecules with the use of specific antibody reagents that can detect the reactive metabolites when they are covalently bound to proteins. This approach has allowed the characterization of new protein targets of reactive and toxic metabolites of the inhalation anesthetic halothane, the nonsteroidal anti-inflammatory agent diclofenac, the anti-hypertensive drug guanabenz, and of nitric oxide, and to begin studying the pathological implications of these covalent alterations.

The inhalation anesthetic halothane continues to be used as a model drug for determining how drugs cause idiosyncratic hepatitis. Last year, it was found that the trifluoroacetyl chloride metabolite of halothan covalently altered a 170 kDa protein in the endoplasmic reticulum of rats. The protein was identified as UDP-flucose:glycoprotein glucosyltransferase, an enzyme that is involved in the formation of the correct conformation of glycoproteins. It was also found that the activity of the enzyme was inhibited by treatment of rats with halothane, suggesting that the covalent alteration of UDP-flucose:glycoprotein glucosyltransferase might be a factor contributing to the development of halothane hepatitis. In other studies, halothane hepatitis patients were found to have serum autoantibodies that reacted with a purified human liver 58 kDa protein that was cloned from a human live cDNA library and expressed in milligram quantities with the use of a baculovirus expression system. This finding supports the idea that halothane hepatitis has at least in part an immunopathological basis. Moreover, this year the group rported the first case of hepatitis cause by the newest inhalation anesthetic, desflurane, and has provided evidence that the toxicity might have been caused by a cross-sensitization reaction, due to previous exposure and sensitization to halothane.

The group has continued to study the protein adducts of nonsteroidal anti-inflammatory agents, in an effort to determine how this widely used class of drugs causes hepatitis. This year, the 110 kDa plasma membrane protein target of the nonsteroidal anti-inflammatory drug diclofenac has been identified from rats as dipeptidyl peptidase IV, which is identical to the lymphocyte accessory molecule, CD26. The enzyme was inactivated by diclofenac, suggesting a possible mechanism of hepatotoxicity. In addition, a 50 kDa protein target of diclofenac was identified as P450 2C11, an enzyme that has been associated with an immune-based hepatitis cause by another drug, tienilic acid.

In another project area, this group has studied the involvement of nitric oxide synthase (NOS) and its product nitric oxide in various pathological conditions. This year, it was discovered that the anti-hypertensive agent guanabenz irreversibly inactivated NOS in penile tissues of rats, suggesting a possible mechanism by which this agent and possibly other anti-hypertensive drugs cause impotence. Moreover, it was found that nitric oxide can lead to the nitration of tyrosine residues of proteins in synovial tissue of arthritis patients, which may have a role in the development of arthritis.

E. Neurotoxicity in dopamine containing neurons, regulation of dopamine uptake and release, and programmed cell death in neuronal cultures

The excitotoxic effects of kainic acid, AMPA, and N-methyl-D-aspartate were studied in mesencephalic primary cultures as part of a longer term interest in understanding the role played by selective vulnerability of neurons in the pathophysiology of Parkinson's disease and stroke. Dopaminergic neurons, which comprise about 5% of the total cell population, are more vulnerable to glutamate receptor stimulation than calretinin-containing neurons. A 24 hour exposure to 500 μM kainic acid reduced the number of dopamine-containing neurons by 40% but failed to change the

number of calretinin-containing neurons. The calretinin-containing neurons are injured after 48 hours of exposure to kainic acid. Immunohistochemical studies showed theat the GluR2/R3 receptor subtype was present in both cell types, and both cell types could respond to GluR2/R3 receptor stimulation. The studies are consistent with the possibility that calretinin plays a $[Ca^{2+}]_i$ -buffering role that confers resistance to glutamate neurotoxicity.

In primary cultures of embryonic mesencephalic neurons, nitric oxide (NO) facilitates dopamine release in a dose-dependent manner by a Ca^{+}-independent mechanism. In the same neuronal cultures, addition of NO to the incubation medium revesibly inhibited dopamine uptake. Since NO can react with biomolecules characterized by one-electron reduction potentials, such as ascorbate, L-glutathione, $O_2^{\cdot}$, or dopamine, the chemical interaction of NO with dopamine was studied in absence or presence of antioxidants and O_2. NO facilitated the formation of dopamine o-semiquinone under aerobic but not anaerobic conditions and was abrogated by the presence of ascorbate or L-glutathione. The inhibition of ^{3}H-dopamine uptake was not abated by the presence of ascorbate or L-glutathione suggesting that NO may interact directly with a cellular target that regulates vesicular storage of dopamine.

It was also found that DNA fragmentation occurred in absence of necrosis when the mesencephalic neuronal cultures are deprived of neurotrophic factors. In this condition, the exposure to Pb^{2+} increases the appearance of neuronal necrosis. In contrast, the exposure of cerebellar granule cell cultures to Pb^{2+} increases the density of DNA fragmentation. This process is accelerated when the availability of neurotrophic factor is decreased.

F. Role of nitric oxide in retinal function and disease.

In retina, cyclic GMP plays a major role in light transduction. Nitric oxide (NO) is known to activate guanylate cyclase in a variety of tissues, but this has not been well studied in retina. Previous studies indicated that human retina expresses both the constitutive brain form of nitric oxide synthase (NOS) and the inducible form. For the first time, full length NOS cDNAs have now been characterized from human retina. The constitutive form of retinal NOS was transfected into CHO cells and found to be calcium-calmodulin dependent and to require NADPH. The Km for arginine was 4.4 μM and the enzyme was inhibited by N-nitroso L-arginine in a competitive fashion. The same human retinal cDNA is being expressed in Drosophila to investigate the role of nitric oxide in learning and memory. These studies are in part motivated by studies by other labs indicating that two genes essential for memory and learning encode a cAMP specific phosphodiesterase and a calcium-calmodulin sensitive adenylate cyclase. The group wishes to investigate the potential role of cGMP in learning and memory and therefore is studying NO as a stimulator of cGMP. Two forms of cDNAs for i-NOS have also been identified from human retina. The cDNAs differ, apparently due to alternative splicing. Plans are to investigate the roles of both constitutive and inducible forms of NOS in retinal function and disease.

ANNUAL REPORT OF THE
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Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that support and control transport and metabolism.

Studies in single renal tubules

Studies were completed by Nielsen, Chou, Kishore, and Knepper along with D. Marples and E.I. Christensen of Aarhus University (Denmark) which demonstrated in isolated perfused renal collecting ducts that exposure to vasopressin causes a marked redistribution of aquaporin-2 water channels from intracellular vesicles to the apical plasma membrane, thus accounting for the vasopressin-induced increase in the water permeability of the collecting duct cells. These studies provide a direct verification of the so-called "shuttle hypothesis", i.e. regulation of plasma membrane water permeability by regulated exocytosis of vesicles containing water channels. Nielsen and Knepper in collaboration with Marples and Christensen (Aarhus, DK) reinforced this conclusion by demonstrating that in vivo stimulation of collecting ducts by vasopressin in rats induces a shift of inner medullary water channels from a membrane fraction enriched in intracellular vesicles (high speed pellet) to a membrane fraction enriched in plasma membranes (low speed pellet). Experiments by Nielsen and Knepper along with Marples, Dalby, Birn, and Motashami (all of Aarhus, DK) demonstrated that a vesicle targeting protein, VAMP-2 (or synaptobrevin-2) colocalizes with aquaporin-2 in intracellular vesicles of collecting duct cells. A related protein VAMP-1 was not found in kidney tissue. As part of the neurotransmitter release mechanism in the brain, this protein is proposed to bind to a cognate receptor in the target plasma membrane, viz. a syntaxin, to mediate specific docking of the vesicles to the correct target membrane. Mandon, Nielsen, and Knepper have localized mRNAs for the four non-Golgi syntaxins in the kidney using RT-PCR in microdissected glomeruli, renal tubules, and blood vessels. These data indicate that syntaxin-2, -3 and -4, but not syntaxin-1, are expressed in various structures of the kidney. Messenger RNAs for only syntaxin-3 and syntaxin-4 were found along the entire collecting duct system. Because recent studies indicate that only syntaxin-1 and syntaxin-4 bind VAMP-2, we carried out further studies using an antibody that we raised against syntaxin-4 to localize the syntaxin-4 protein. Immunoblotting revealed a protein of the correct apparent molecular mass (36 kDa) in the inner medulla, with demonstrated enrichment of the protein in isolated inner medullary collecting ducts relative to whole inner medullary suspensions. Immunocytochemistry (immunoperoxidase)

revealed localization to the apical plasma membrane, consistent with its proposed role in targeting of aquaporin-2 water channel containing vesicles translocated to the apical membrane in response to vasopressin.

Olson, Goldsmith, and Elliot (NICHD), in collaboration with Knepper, developed a method for quantification of aquaporin-2 water channel excretion in human urine using immunoblotting with aquaporin-2 standards. Initial studies demonstrated that intact aquaporin-2 can be measured in human urine and that the rate of aquaporin-2 excretion increases markedly in response to acute elevation of plasma vasopressin levels induced by hypertonic saline infusion, raising the possibility that measurements of urinary aquaporin-2 excretion could be used as a clinical indicator of acute vasopressin response in the renal collecting duct.

Ecelbarger, Terris, Nielsen, and Knepper, along with Marples (Aarhus, DK), have raised a peptide-directed antibody against the renal water channel aquaporin-3 from rat and used it for localization studies in the rat kidney using immunoblotting and immunocytochemistry. In the kidney, aquaporin-3 is found exclusively in the basolateral domain of collecting duct principal cells in cortex, outer medulla, and inner medulla. Virtual absence in intracellular vesicles suggests an absence of rapid regulation by vesicle shuttling (compare to aquaporin-2 above).

Terris, Ecelbarger, Nielsen, and Knepper, along with Marples (Aarhus, DK), have also raised a peptide-directed antibody against the renal water channel aquaporin-4 from rat and used it for localization studies in the rat kidney using immunoblotting and immunocytochemistry. In the kidney, aquaporin-4 is found exclusively in the basolateral domain of the collecting duct, but unlike aquaporin-3, it is limited to the inner medullary and outer medullary collecting ducts. As with aquaporin-3, absence in intracellular vesicles suggests an absence of rapid regulation by vesicle shuttling.

Studies were completed by DiGiovanni, Nielsen, Christensen, and Knepper demonstrating that long-term elevation of circulating vasopressin levels is associated with a marked increase in expression of the aquaporin-2 water channel in the renal collecting ducts of Brattleboro rats, providing an explanation for long-term regulation of water permeability in the renal collecting duct. Current studies by Terris, Ecelbarger, Nielsen, and Knepper using immunoblotting have demonstrated that the expression of both aquaporin-2 (the apical water channel) and aquaporin-3 (a baso-lateral water channel) is increased in renal medulla and cortex by in vivo water restriction or by 5-day vasopressin infusion. The effect of vasopressin was not eliminated by the elimination of countercurrent multiplication by simultaneous infusion of furosemide, suggesting that the effect of vasopressin is not dependent on increases in interstitial osmolality in medulla and cortex. Studies by Olson and Elliot (NICHD) and Verbalis (Georgetown), in collaboration with Ecelbarger and Knepper, showed that marked suppression of aquaporin-2 expression in the rat inner medulla can be induced by water loading in the setting of chronic administration of the vasopressin analog dDAVP. These experiments indicate that

long-term regulation of water channel expression can occur independent of vasopressin and that downregulation of aquaporin-2 expression could be involved in vasopressin escape.

Several studies have investigated the possible role of dysregulation of aquaporin water channel expression in several pathophysiological processes associated with decreased urinary concentrating capacity. Nielsen and Knepper, along with Marples (Aarhus, DK), have demonstrated marked downregulation of inner medullary aquaporin-2 water channel expression associated with both hypokalemia and chronic lithium administration. Similarly, Knepper and Nielsen, along with Marples and Froekiaer (Aarhus, DK), have demonstrated a similar suppression of inner medullary aquaporin-2 expression in bilateral ureteral obstruction. Finally, Apostol, Terris, Ecelbarger, and Knepper, in collaboration with Andrews (Georgetown), demonstrated a similar suppression of aquaporin-2 and aquaporin-3 expression in the rat inner medulla associated with puromycin aminonucleoside-induced nephrotic syndrome. We propose that downregulation of water channel expression is a key factor in the decreased concentrating ability found in all of these syndromes and that a common downregulatory mechanism may be involved (see below).

Kishore and Knepper continued work on development of methods to quantify expression of specific proteins in microdissected tubules using specific polyclonal antibodies in a fluorescence-based ELISA. The approach was successfully employed for measurement of aquaporin-1 and aquaporin-2 expression levels in single tubules and vasa recta segments. Aquaporin-1 levels in descending vasa recta were found to be sufficient to account for measured water permeabilities by Pallone (Hershey). Expression levels of aquaporin-2 were found to be very high (>1 million copies per cell) throughout the collecting duct system. Surprisingly, similar levels were found in the renal connecting tubule arcades, which are believed to be impermeable to water. Studies of the arcades were extended by Mandon, Kishore, Wade (U. of Maryland), and Knepper who showed: 1) using RT-PCR that microdissected arcades contain high levels of mRNA for both the aquaporin-2 water channel and the V2 vasopressin receptor, and 2) using immunocytochemistry that aquaporin-2 colocalizes with two connecting tubule markers, kallikrein and the sodium-calcium exchanger protein. These studies support the possibility that the connecting tubule arcade may be a site of vasopressin regulated water transport.

Knepper, Smith, and Hediger (Harvard) cloned the vasopressin-regulated urea transporter (UT2) cDNA from rat. Terris, Nielsen, and Knepper prepared peptide-directed polyclonal antibodies using the predicted amino acid sequence from the rat cDNA and used one of these antibodies for cellular and subcellular immunolocalization in rat kidney. The antibody identified a single 97 kDa band on immunoblots using membranes from rat inner medulla. Immunocytochemistry revealed labeling of the apical region of inner medullary collecting duct cells and immunoelectron microscopy (immunogold) showed labeling of the apical plasma membrane and

intracellular vesicles, but not the basolateral plasma membrane. The presence in intracellular vesicles raise the possibility that the vasopressin-regulated urea transporter, like the vasopressin-regulated water channel aquaporin-2, may be translocated into the apical membrane by regulated exocytosis in response to vasopressin.

Studies were completed by Kishore, Chou, and Knepper showing that occupation of nucleotide receptors (P2u purinergic receptors) in the basolateral plasma membrane of inner medullary collecting duct cells by ATP or UTP results in marked inhibition of epithelial water permeability. These studies show that the inhibition is associated with a decrease in cyclic AMP levels in the cells and that the effect is dependent on activation of protein kinase C. Studies by Chou and Knepper demonstrated activation of protein kinase C activity in an inner medullary collecting duct suspension by carbachol and by ATP. Immunoblotting studies revealed that the predominant isoforms in IMCD cells are delta and epsilon, both of which are calcium independent isoforms. Subcellular fractionation studies in IMCD cell suspensions show translocation from the cytosolic fraction to the membrane fraction in response to carbachol or the phorbol ester, PMA. These studies support that possibility that protein kinase C activation in IMCD cell could be involved in short-term and long-term regulation of water permeability.

Transport in model epithelia

Kovbasnjuk, Leader, Xia, and Spring have utilized optical microscopy to study the composition of the fluid filling the intercellular spaces between epithelial cells. They studied cultured renal cells (MDCK as well as LLC-PK_1), grown on glass coverslips or permeable supports, to measure the Na and Cl of the spaces between the cells and to determine the diffusion coefficient of fluorescent dyes and ions within the intercellular spaces.

Organic osmolytes

Bacterial, plant, and invertebrate animal cells are known to accumulate compatible, osmotically active, organic intracellular solutes when their environment becomes hyperosmotic. These organic "osmolytes" help maintain the intracellular milieu because they do not perturb vital intracellular macromolecules, in contrast to sodium and potassium salts which in abnormally high concentrations do perturb macromolecules. Most mammalian body fluids are not normally hyperosmotic and the cells exposed to them do not normally express organic osmolytes. The exception is the renal inner medulla in which the interstitial fluid is hyperosmotic to a variable extent because of the renal concentrating mechanism. We identified large and variable amounts of organic osmolytes in rat and rabbit inner medullary cells, namely sorbitol, inositol, glycerophosphorylcholine (GPC), taurine, and betaine.

Control of the cellular accumulation of these osmolytes is most readily studied in tissue culture. We screened several renal cell lines in hyperosmotic media and found that cells that survived accumulated the same organic osmolytes previously found in intact renal medullas. These cell lines are now being studied in detail. The findings with regard to osmotic regulation of the individual organic osmolytes are, as follows:

1. Sorbitol accumulates in GRB-PAP1 cells by synthesis from glucose, catalyzed by aldose reductase. Hypertonicity increases aldose reductase gene transcription, mRNA abundance, and protein abundance. We have cloned the rabbit aldose reductase gene and are testing the 5' flanking region in a transient expression system to determine the molecular mechanism by which hypertonicity stimulates transcription of this gene.

2. Betaine is accumulated by MDCK cells in hyperosmotic medium because of increased transport into the cells. We cloned the cDNA for the renal cell betaine transporter by expression in toad oocytes. Using the cDNA as a probe, we find increased expression of the betaine transporter in MDCK cells exposed to hypertonic medium. The cloned betaine transporter has a nucleotide sequence similar to several recently cloned rat brain transporters which constitute a new transporter family. Current studies are investigating the effect of accumulation of betaine and the other osmolytes on expression of the betaine transporter. Also, we have cloned the mouse renal medullary betaine transporter cDNA.

3. GPC. In contrast to the other osmolytes, GPC accumulation is triggered by high urea, as well as by high NaCl. Accumulation of GPC by MDCK cells in hyperosmotic medium is due to increased net synthesis. Choline is an essential precursor. The choline is incorporated into phosphatidylcholine, which is hydrolyzed to form GPC. Activities of the enzymes involved in GPC synthesis (phospholipase A_2) and degradation (GPC: choline diesterase) are being measured. Depending on the conditions, the phospholipase may be activated and/or the diesterase may be inhibited to increase GPC; effects of urea and NaCl differ. NaCl and urea added together inhibit GPC:choline diesterase. We have purified GPC:choline diesterase, and derived some of its amino acid sequence. Present work is aimed at cDNA cloning of this enzyme and preparation of antibodies against it as steps toward determining how hyperosmolality controls its activity. Also, we are studying these enzymes in renal medullas of rats treated in various ways in order to elucidate the role of the enzymes in vivo.

4. Identification of other genes that respond to osmotic stress. In addition to the genes involved in accumulation of organic osmolytes, hypertonicity is known to increase the expression of early response genes and genes coding for heat shock proteins. There presumably are other genes that are also important for osmotic regulation that are as yet unknown. In order to find them we are employing the technique of differential display to identify mRNAs whose abundance is greater in MDCK cells exposed to high NaCl. Several different cDNAs have been cloned that

are induced by hypertonicity. We have sequenced and studied osmotic regulation of expression of one of them, namely CD9.

Accumulation of organic osmolytes in response to osmotic shock is a basic biological phenomenon previously identified from bacteria to cells in lower vertebrates. The present recognition of its vital role in renal medulla is the first indication that it is more than a curiosity in mammalian cells. Possible disorders of this system have not yet been investigated, but there are a number of poorly understood diseases of the renal medulla that should be considered. Further, the aldose reductase system, whose function we are unravelling in the renal medulla, is implicated in complications of diabetes in eyes, nerves, and kidneys.