NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT
INTRAMURAL RESEARCH PROGRAM

ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR
1986

epartment of Health and Human Services
Health Service
l Institutes of Health

TABLE OF CONTENTS

INTRAMURAL RESEARCH PROGRAM

NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT (U.S.)

1986 ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR

RJ
85
N277
1986
pt.4

NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT
INTRAMURAL RESEARCH PROGRAM

ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR
1986

The Intramural Research Program is broadly concerned with the biological and neurobiological, medical and behavioral aspects of normal and abnormal human development. In addition to four major clinical research and training programs in the areas of genetics and endocrinology, a diversity of developmental models are under study in twelve fundamental research Laboratories, drawing upon observations in bacteria, Drosophila, yeasts, viruses, molluscs, frogs, rodents, and subhuman primates. Disciplines employed in these studies include biochemistry, virology, molecular biology, immunology, pharmacology, genetics, cell and neuronal biology, biophysics, mathematical and theoretical biology, reproductive physiology, and comparative ethology.

During the past year, we have continued to strengthen a number of research programs that we believe to have a particular promise and timeliness. In these areas, new resources have been added so that the investigators will be optimally poised to exploit their recent findings. These programs address questions in cell and molecular biology, wherein we anticipate significant results with broad implications for biomedical research. One topic of great interest is being pursued by the Cell Biology and Metabolism Branch, which focuses on the developmental and regulatory aspects of various cellular organelles and receptors. Investigators in this Branch are studying structure-function relationships as well as molecular regulation of interleukin-2 (T cell growth factor) receptors. A similar approach is being taken to studies on the T cell antigen receptor. Both types of receptors influence the proliferation and activity of immunocytes, and anomalies in their structure or regulation may underlie many immunological disorders.

The Laboratory of Molecular Genetics is expanding its interest in homeotic genes, which specify the spatial and temporal parameters of organ and limb morphology during development of the fruit fly, Drosophila melanogaster. Recently, it has become apparent that these very important genes, which play a critical role in normal embryogenesis, have been highly conserved through evolution, and homologous genes appear to be present in humans. The LMG is also continuing to expand its work in yeast genetics. Yeast is a eukaryotic organism which offers a level of genetic and molecular analysis that is difficult to achieve in higher eukaryotes. Moreover, many essential genetic mechanisms and biochemical pathways manifested in the human are demonstrable in this organism. Much emphasis has also been given to work on the transgenic mouse in the LMG. This work has flourished during the past year, and many new transgenic mouse strains have been established, displaying the addition or deletion of heritable characteristics. The transgenic mouse model presents extraordinary opportunities for the study of tissue-specific temporal gene regulation, particularly during mammalian development, but the work also serves as a prelude to gene therapy in humans.

An area that has received much recent attention, in both the Laboratory of Developmental Neurobiology and the Laboratory of Neurochemistry and Neuroimmunology, relates to the cell biology of brain development, especially the hormonal and electrical phenomena that endow the young mammalian brain with its rich but still not well-appreciated structural and functional plasticity. Tools have also become available to apply classical molecular biological techniques to the study of gene regulation in the nervous system, and we plan to pursue this area vigorously.

In clinical research, the Human Genetics Branch has been intensifying its efforts in the diagnosis and treatment of human genetic diseases, especially osteogenesis imperfecta and various inborn errors of metabolism such as cystinosis. Here too, the availability of powerful new molecular and cell biologic tools is permitting our investigators to illuminate the underlying mechanisms of these diseases and to devise new therapies.

The Laboratory of Developmental and Molecular Immunity has expanded both its applied and basic studies. Much effort has gone into the development of bacterial vaccines, the design of which is predicated upon an understanding of the development of the immune system in infants and children. In particular, the group responsible for these efforts has focused on new vaccines directed against pertussis, H. influenzae, Salmonella typhi, and meningococcus--all agents with great consequences for the public health. At the more basic level, the LDMI has moved heavily into studies on the molecular regulation of genes which influence important immunological phenomena such as the expression of class I mixed histocompatibility (MHC) antigens, and the interferon system.

A number of new areas in endocrinology have received particular attention during the past year, again ranging from the applied to the basic. At the more applied level, the Laboratory of Theoretical and Physical Biology has expanded its efforts in the development of algorithms and computer programs that guide the therapeutic management of chronic disorders such as diabetes. The programs have the potential to greatly refine treatment regimens, and also encourage the patient with access to a computer to take a more active role in his or her own treatment. The national data base being accrued through the use of these programs is clearly an invaluable clinical research tool. At the more basic level, much new effort in the Endocrinology and Reproduction Research Branch, as well as the Developmental Endocrinology Branch, has been devoted to cell biologic and molecular studies which exploit the growing awareness that hormones and releasing factors, formally thought to have roles only within the endocrine axis, in fact have far broader roles, particularly with respect to the integration of global brain responses such as occur in stress, hypertension, and depression. In clinical research, the Developmental Endocrinology Branch is assessing the new generation of therapeutic hormones, e.g., growth hormone, produced by recombinant DNA technology, as well as novel glucocorticoid and progesterone antagonists which block binding of the endogenous hormones to their receptors.

The Laboratory of Comparative Ethology, established late in 1983, continues to undergo a major building program. An extensive outdoor facility for free-ranging primates, employed in observational studies on the genetics of behavior, is now in place, and construction of a three-floor indoor facility, including breeding quarters and a newborn nursery, has been recently completed.

These facilities are located at the NIH's Animal Center in Poolesville, Maryland.

The Laboratory of Developmental Pharmacology, which has (for almost two decades) focused on the cytochrome P450-associated enzyme system (highly conserved from prokaryotes to man), is fast approaching the development of molecular-biology based assays for determining the individual genetic polymorphisms which influence the function of this system. The P450 enzymes are responsible for the metabolism and detoxification of endogenous ligands as well as countless environmental chemicals which can cause birth defects, cancers, and idiosyncratic drug responses. Intensive effort is being put into the cloning and sequencing of the many genes involved in the P450-system, so as to develop useful probes for individual risk assessment, e.g., the risk of lung cancer faced by a given cigarette smoker.

Finally, the laboratories of the Scientific Director and Deputy Scientific Director have expanded their studies on growth factors, especially nerve growth factor (NGF) and transforming growth factors (TGF's), as it has become increasingly evident that these factors may play critical but complex roles in normal development as well as such diseases as cancer and chronic neurological disorders. Much effort is also being directed to studies on the mechanism of mammalian mutagenesis, taking advantage of new recombinant DNA tools such as "shuttle vectors."

The Institute continues to acquire additional laboratory space. Construction for a three-floor addition to Building 6 is almost completed, and will provide twenty-five new laboratory modules, as well as an extensive animal facility. This new space will accommodate the Laboratory of Developmental Pharmacology, one section of the Laboratory of Developmental and Molecular Immunity, and the DNA/protein Sequencing and Synthesis Unit of the Endocrinology and Reproduction Research Branch. When these moves are complete, we will be able to move the Laboratory of Theoretical and Physical Biology into larger, centralized quarters, and as well, we shall increase the amount of laboratory space assigned to a number of other Laboratories and Branches located in the Clinical Center, including the Human Genetics Branch, the Developmental Endocrinology Branch, and the Endocrinology and Reproduction Research Branch. Within the neuroscience complex (Buildings 36 and 37) we have recently added twenty additional modules for neurobiology research. The space is now being renovated and should be ready for occupancy in 1987. Plans are underway also for a large additional structure annexed to Building 6 (Bldg. 6 "B") that would house a large transgenic mouse facility, as well as additional laboratory and animal space. Finally, the Congress has appropriated $5 million in 1987 for the design of a new building ("49") on the NIH campus that would house elements of the NICHD (especially neuroscience laboratories), as well as neuroscience laboratories of other Institutes and a campus-wide primate facility. Assuming that Congress will appropriate construction funds for "Building 49," this Program will have grown from 41,000 square feet of laboratory space (in 1983) to 110,000 square feet by 1990.

In major personnel changes during the past year, Dr. William Gahl was granted tenure in the Human Genetics Branch and Dr. Fernando Cassorla in the Developmental Endocrinology Branch. Dr. Richard Knazek transferred to the Developmental Endocrinology Branch from the NCI, and Dr. Robert Klein left the Laboratory of Comparative Ethology to join the Division of Computer Research Technology.

Our clinical and laboratory research fellowships for physicians in adult, pediatric, and reproductive endocrinology, as well as the fellowship in medical genetics, continue to thrive, and in the past year, we have developed new sources of support for post-doctoral (Ph.D. and M.D.) fellows. These funds include post-doctoral stipends paid by the Institute but which do not encumber regular government positions. We anticipate that these new mechanisms will greatly enhance our ability to offer post-doctoral training. The new mechanisms include an Intramural National Research Scholarship Award (NRSA) program for M.D.'s with a Public Health Service pay-back obligation, a contractual program for the support of intramural training in biotechnology administered by the National Research Council of the National Science Foundation, and a new fellowship program which began October 1, 1986 that is intended for American post-doctoral candidates (Ph.D.s or M.D.s). The latter mechanism is the domestic equivalent of our long-standing Visiting Fellow Program for post-doctoral candidates from abroad. The new program (Intramural Research Training Award, or "IRTA") will only be limited by available funds. We have also been successful in identifying new donors of stipends, including endowments by private industry and foundations. NICHD funds administered by the NIH's Fogarty International Center have been employed for the support of sabbatical visits by senior scientists from abroad. Moreover, a number of foreign post-doctoral fellows have been awarded stipends for training in our laboratories under the terms of formal bilateral agreements generated between the NIH and several countries in Europe, Asia, and the Middle East. Finally, a number of medical students supported by the new Howard Hughes Foundation program at the NIH are working in our laboratories during an elective year, and our Summer Student Program continues to be very successful, with more than fifty undergraduate, graduate, and medical students working in our laboratories this year (most were here on a volunteer basis).

Peer review of intramural research, conducted by the Institute's Board of Scientific Counselors and ad hoc experts, continues to receive great emphasis, with rigorous site visits to each Lab at four year intervals. During the past year, visits were made to the Human Genetics Branch, the Laboratory of Neurochemistry and Neuroimmunology, and the Laboratory of Developmental and Molecular Immunity, with detailed critiques prepared as a consequence of these visits. The membership of the Board of Scientific Counselors reflects the increasing diversity of research interests within this Intramural Program. The current Board membership includes:

Joseph G. Gall, Ph.D., Senior Member, Department of Embryology, Carnegie Institution of Washington (Chairman)
Stanley Cohen, Ph.D., Professor, Department of Biochemistry, Vanderbilt University*
John C. Marshall, M.D., Ph.D., Professor, Department of Medicine, University of Michigan

---

* We were delighted to learn in October that Stanley Cohen, together with Rita Levi-Montalcini, won the 1986 Nobel Prize in Medicine or Physiology for their discovery of Nerve Growth Factor.

Lewis P. Lipsitt, Ph.D., Professor, Department of Psychology, Brown University
Allen H. Neims, M.D., Ph.D., Professor and Chairman, Department of Pharmacology and Therapeutics, University of Florida
Story C. Landis, Ph.D., Professor, Department of Neurobiology, Case Western Reserve University
Merry R. Sherman, Ph.D., Professor, Department of Biochemistry, Rutgers University
Harold Amos, Ph.D., Professor, Department of Bacteriology and Immunology, Harvard Medical School
John Phillips, Jr., M.D., Professor, Department of Human Genetics, Vanderbilt University School of Medicine

Other developments in the past year include the continued strengthening of all aspects of the management of animals employed in our research. Dr. John Donovan, the Institute's veterinarian, left during the year to become Chief Veterinarian for the National Cancer Institute, but we have been fortunate to recruit Dr. William Stokes from the U.S. Army Medical Research Command to fill this position. We have continued to develop new computer-based administrative procedures in the Office of the Scientific Director so as to maximize the efficiency with which our resources are deployed. These new administrative approaches are ensuring the maximum yield with respect to scientific productivity while the current climate of constrained resources persists.

Seminars by the twelve Laboratories and Branches in this Program were numerous and well attended throughout the year, such that this Institute organized a relatively large fraction of the NIH's overall offering of intramural seminars and workshops. During the past year also, three major international conferences were organized by Laboratories of the Intramural Research Program on the Bethesda campus, including·

The Molecular Genetics of Development in Frogs, Flies, and Mice (Laboratory of Molecular Genetics)
Mechanisms of Physical and Emotional Stress (Developmental Endocrinology Branch)
Advances in Research on Human Growth (Developmental Endocrinology Branch)

Another meeting, on developmental biology, was organized by this intramural research program and the Centre National de la Recherche Scientifique, and held in Paris during October; speakers included Drs. Dawid, Ozato, Westphal, Nelson, and Levine. During the coming year, in honor of the NIH's centennial, we plan major conferences on the Cytochrome P450 System, Neuronal Plasticity, the Molecular and Developmental Regulation of the Immune System, and the Biochemistry of Receptor Activation.

During the year, we were especially honored by the choice of Dr. Igor Dawid (Chief, Laboratory of Molecular Genetics) to present the NIH's Annual Mider Lecture, one of only two named lectureships at this Institution. Dr. Dawid was also awarded the Presidential Meritorious Executive Rank Award for his exceptional role as a scientific leader. We have also been honored by the award to Dr. Daniel Nebert (Chief, Laboratory of Developmental Pharmacology) of the Bernard Brodie Award of the American Society for Pharmacology and Experimental Therapeutics in recognition of the landmark work that Nebert has accomplished on the pharmacogenetics of the cytochrome P-450 system. Dr. Heiner Westphal

received the Public Health Service's Superior Service Award for his exceptional productivity in studies on the transgenic mouse. Dr. David Rodbard received the Public Health Service's Meritorious Service Medal for his development of algorithms that have improved the management of diabetes and other chronic illnesses. Dr. Richard Klausner also received the Meritorious Service Medal for having provided the first comprehensive view of the molecular regulation of iron metabolism, vital to all aspects of cell viability and proliferation. Dr. David Klein received the NIH Director's Award for his body of work on the structure and function of the pineal gland and the regulation of its important biosynthetic pathways. Dr. John Donovan was awarded the PHS's Commendation Medal for his leadership in NIH animal care issues. Additionally, many of the Institute's Senior Investigators held honorary lectureships and visiting professorships during the year; major prizes for research accomplishments were awarded to a number of our scientists by various universities and societies.

Finally, we are currently sponsoring two distinguished senior scientists in the Fogarty Scholars-in-Residence Program, Professor Donald Brown of the Carnegie Institution (Washington) and Professor Itzhak Parnas of the Hebrew University (Jerusalem), who are working in our Laboratories during their sabbaticals.

In December of 1982, this Institute embarked upon an ambitious program designed to improve the quality and quantity of its intramural scientific productivity, even as the rate of growth in research support declined. As one reflection of our success in this regard during the past four years, NICHD intramural scientists this year published more than twice the number of peer-reviewed original scientific reports, appearing in journals of stature, than had been the case prior to the beginning of this new direction. Moreover, one out of every two of our post-doctoral scientists is now fully supported by a stipend awarded from a non-NIH source--reflecting, we believe, the current quality and productivity of this Intramural Program.

With regard to the major research interests and scientific results during the past year, particularly notable are the findings described on the following pages.

Laboratory of Molecular Genetics--
Igor Dawid, Ph.D., Chief

Investigators in this Laboratory use the tools of molecular and cellular biology to answer questions about gene transmission and recombination, and the regulation of genetic functions during development. The range of model systems under investigation includes bacterial and animal viruses, transformed animal cells, yeasts, mouse and Xenopus embryos, and the fruit fly Drosophila melanogaster. Although this diversity of model systems is broad, the Laboratory relies on a commonality of basic interests, conceptual approaches and technologies. Recombinant DNA and gene transfer methods are emphasized. The development of novel vectors is being pursued for the introduction and expression of isolated genes in animal cells.

During the past year, the members of the Laboratory have continued to utilize the methods of molecular biology to gain insight into mechanisms of regulation of the genetic material, especially in response to environmental stimuli such as the nutritional state and during the multifaceted events of differentiation. Different biological systems were studied, each with its unique characteristics, special points of interest, and particular suitability for answering specific questions. Advances have been made in many areas. Particularly interesting are results obtained in the study of transgenic mice which highlight the correlation between the targeted expression of an oncogene and tissue-specific tumorigenesis, as well as the relationship between transformation and differentiation. Other significant results concern the mechanism of translational regulation of a yeast protein that controls transcription, offering the first detailed insight into a novel and probably general type of regulatory network in a eukaryotic cell.

Gene regulation in Transgenic Mice

Heiner Westphal and his colleagues have established in the Laboratory the methodology of inserting genetic material into the mouse germline to produce transgenic mice. Fertilized eggs, micro-injected with any DNA of interest, are carried to term in surrogate mothers. In general, this methodology yields results in three different areas, all of which are represented in the work of this group: (i) The determination of the control regions of individual genes that are required for temporal and tissue-specific regulation in the whole animal; (ii) insertion of genes that have a dominant effect in the transgenic animal; and (iii) production of mutants through insertional mutagenesis. Results relevant to point (i) have been reported in the previous Annual Report; the clearest result in this area, and the one relevant to current work, is the observation, made in collaboration with Joram Piatigorsky and his colleagues in the National Eye Institute, that a region of only 409 nucleotides from the 5' region of the αA-crystallin gene can direct the proper expression of a marker gene (chloramphenicol acetyltransferase, CAT) in the transgenic mouse. CAT activity was found only in the lens fiber and epithelial cells beginning at 12.5 days of gestation, showing precise temporal and spatial regulation of the αA-crystallin/CAT fusion gene.

The results summarized above define a highly specific promoter element, the properties of which were used to target the expression of an oncogene to a specific tissue, with the aim of producing a dominant phenotype in a transgenic strain. The SV40 T antigen gene (the transforming gene of this DNA tumor

virus) was fused to the αA-crystallin promoter and inserted into the germ line. The resultant progeny reproducibly exhibited bilateral cataracts as soon as the newborns opened their eyes. Histological studies employing standard staining, as well as immunocytochemistry and in situ hybridization with nucleic acid probes, showed that the lenses of these animals were filled with transformed cells. Such cells stained for both T antigen and crystallins, but crystallin staining was patchy. Transformed cells were first detectable at the time of initial crystallin expression; thus, it appears that as the lens cells begin to differentiate, crystallin genes are turned on, and with them, the hybrid crystallin/T-antigen genes. Very shortly after this time, the phenotypic effects of transformation become detectable. The affected mice usually die at an age of 3 to 4 months. However, removal of the eyes at birth appears to prolong their lifespan; one such operated animal died at 6 months with evidence of multiple tumors, all of which expressed the SV40 T antigen. These results are of considerable interest from several points of view, among them the generation of lens tumors--which do not occur under natural conditions in any animal including humans. Further, the time course of transformation in a differentiating tissue, and the apparent suppression of the differentiated phenotype by transformation (reduction in crystallin expression), can be studied both in vivo and in vitro in cells cultured from the affected lens.

Observations relevant to insertional mutagenesis have also been made. Two dominant traits and one recessive trait have been observed among the transgenic mice studied so far. The dominant trait in both cases is small litter size, which appears by cytogenetic analysis to be due to chromosomal translocations. One translocation which has been studied in detail is linked to the inserted foreign DNA, and appears to have been precipitated by the insertional event. The recessive trait is syndactyly; again, it may be hypothesized that the mutation has been generated by the insertion of the foreign DNA into a locus involved with limb formation. The great advantage of mutations generated through DNA insertion, as opposed to more traditional mutagens, is that the introduced DNA and the regions flanking it can be isolated from a library of the mutant genome by virtue of the available transgenic sequence. Such an approach might allow the study of developmentally interesting genes (i.e., the DNA flanking the "probe") with no known products in a mammalian species.

In sum, the results obtained demonstrate that the transgenic mouse system provides an approach to the study of gene regulation and developmental mechanisms in the mammal that goes well beyond anything possible before the introduction of this new and powerful methodology. Indeed, the transgenic mouse will revolutionize biomedical research as did the advent of tissue culture in an earlier era.

The Mechanism of General Amino Acid Control in Yeast

The group headed by Alan Hinnebusch is concerned with the regulatory pathway in yeast known as "general amino acid control." Yeast is a eukaryotic organism which offers a level of genetic and molecular analysis that is difficult to achieve in higher eukaryotes. A multitude of yeast structural and regulatory mutations have been identified and located on the yeast genetic map, and the cognate wild-type DNA sequences encoding many of these genes have been isolated. Mutations can be introduced into cloned yeast genomes in place of the wild-type sequences. In this way, novel mutations have been constructed and analyzed in vivo in their correct chromosomal locations. It is becoming clear

that yeast cells share with cells of higher eukaryotes a variety of important structures and processes. As a result, it is possible to study these features of eukaryotic cell biology in yeast using a powerful arsenal of molecular and genetic techniques. Moreover, results obtained in yeast should provide useful paradigms to guide studies of analogous processes in higher cells. As an example, the mechanism of coordinate regulation of the transcription of unlinked genes is central to the control of cellular differentiation in higher eukaryotes. The "general amino acid control" found in yeast is a useful model of this type of gene regulation. Hinnebusch and his colleagues have continued their studies on this system that regulates the production of more than 30 amino acid biosynthetic enzymes according to the availability of amino acids in the medium. It has been shown previously that the GCN4 locus is the proximal regulatory element of the many target genes; there is strong evidence that the GCN4 product is a transcriptional regulator of these genes. It has also been shown that GCN4 expression is regulated at the translational level and that several other loci, to be discussed below, are involved in this regulation. The mechanism of GCN4 regulation was explored further in the current year. Four short open reading frames (ORF's) occur in the long leader sequence of the GCN4 mRNA; their total deletion leads to unregulated high expression of the GCN4 product. Point mutagenesis affecting each of the ORF's individually and in groups was carried out and demonstrated that either of the two 3'-proximal ORF's is necessary and sufficient for repression of GCN4. The two 5'-proximal ORF's do not have such a repressing role but rather appear to act positively: They are required for efficient GCN4 expression during starvation. The interaction between the 3' and 5' ORF's is mediated by another locus, GCD1, that acts further upstream in the hierarchy of general control. GCD1 is required for normal repression of GCN4 when amino acids are available; in gcd mutants GCN4 and consequently the general control genes, are constitutively derepressed. This function appears to be mediated through the 5'-proximal ORF's since GCN4 constructs which have only one 3'-proximal ORF are always repressed, even in gcd1 mutants.

The path from an external signal - the presence or absence of amino acids - to regulation of the general control genes is mediated by several loci. GCD1, the locus mentioned above, is just one of a class of genes required for repression of GCN4. A genetic study carried out by S. Harashima and A. Hinnebusch identified four new GCD loci in addition to isolating new alleles in the previously known locus. All five of these genes are required to repress translation of GCN4 under conditions of amino acid availability. Mutations in these five genes have pleiotropic effects, including increased sensitivity to aminoglycosidic antibiotics, suggesting that the wild type gene products are involved in protein synthesis; this makes sense for components that mediate translational regulation. The GCD1 and GCD2 functions are required for progression through the cell cycle.

Mutations in GCD genes overcome the effects of mutations in the positive regulators GCN2 and GCN3, suggesting that these GCN genes act by antagonism of GCD-mediated negative regulation of GCN4. E. Hannig and S. Harashima have found that expression of both the regulatory and temperature sensitive cell-cycle phenotypes of certain gcd1 and gcd2 mutations requires a null allele of gcn3: In a GCN3$^+$ strain, the phenotypes of these gcd mutations are suppressed. Importantly, gcn3-102, which is completely defective for positive regulation of GCN4, behaves more like GCN3$^+$ and suppresses these gcd mutations. The allele specificity of these interactions suggests that the GCN3$^+$ or gcn3-102 proteins

can interact with and stabilize thermolabile gcd proteins. In this model, a complex between GCN3 and GCD proteins is responsible both for coordinating the translational efficiency of GCN4 mRNA with amino acid availability, and for an essential function required for progression through the cell cycle.

The particular interest of this work stems from its detailed analysis of the mechanism of translational control of transcription in a eukaryotic cell. In prokaryotes, modulation of translation of mRNA, while it is still being synthesized on its DNA template, provides for a regulatory mechanism that has been appreciated for some time. The nuclear/cytoplasmic division in eukaryotes makes this type of mechanism impossible; however, the work summarized here provides insight into a mechanism by which translational regulation of a transcriptional activator links the events of protein and RNA synthesis in a regulatory loop.

Gene Expression during Embryonic Development of Xenopus laevis

Igor Dawid, Tom Sargent and their colleagues have continued their work on molecular mechanisms of development in the frog, Xenopus--a superb model for studies of vertebrate development. A set of genes that are expressed for the first time in late blastula to gastrula stages has been used to generate markers for the earliest cell type-specific functions in the embryo. A major focus of recent work has been the family of embryo- and tadpole-specific keratin genes. Keratins are the protein subunits of intermediate filaments in epithelial cells, constituting major components of the cytoskeleton. Structural studies have revealed the remarkable complexity of this gene family in the frog. Seven distinct keratin genes have been isolated in this laboratory and shown to be arranged in three subfamilies. The expression of these genes is limited to embryos and tadpoles while other keratin genes are active in adult skin and other epithelia. These studies have been complemented by 2-dimensional gel electrophoretic analysis of proteins, demonstrating the complexity of the keratin family by another technique. The correspondence between some of the 2-D spots and certain cloned cDNAs has been demonstrated by hybrid arrest translation. The 2-D gel analysis suggests that the major, but not all of the embryonic keratin genes, have been isolated.

The most interesting aspect of the study of keratin gene expression in the embryo concerns the use of these genes in the analysis of ectoderm differentiation. Previously, this laboratory has demonstrated that activation of keratin genes does not require cell-cell interactions but is an autonomous function of embryonic cells. In the current year, detailed studies on the localization of accumulation of keratin mRNA and protein in the embryo were carried out by two complementary techniques, in situ hybridization in sections and immunofluorescence. The former technique had to be modified to allow its effective use in early frog material; advances in this direction have opened up important avenues for the study of amphibian embryogenesis. The experiments show that keratin genes are activated in the outer layer of the ectoderm during the late blastula stage. Keratin filaments begin to form primarily at the outer edge of the ectodermal cells shortly after gene activation; a much looser net of filaments forms in the interior of the outer cells and throughout the inner cell layer of the ectoderm. During gastrulation, the involuting chordamesoderm induces neural development in a specific region of the ectoderm. Prior to contact with the mesoderm, the prospective neural ectoderm does initiate keratin gene expression; however, shortly after contact with the mesoderm, the

accumulation of keratin mRNA and protein ceases and the previously formed material decays. These results lead to the conclusion that keratin gene activation in the embryo is initiated in the ectoderm as a result of preexisting localized information, but subsequent activity is modulated by inductive interactions. Cessation of keratin mRNA accumulation is the earliest available molecular marker for the differentiation of the central nervous system in the amphibian. The use of this marker made possible the conclusion that pre-neural and pre-epidermal ectoderm express some of the same genes which distinguish the ectoderm from endoderm and presumptive mesoderm. Subsequently, probably as a result of inductive influences generated from the chordamesoderm, the neural and epidermal pathways diverge, as reflected in increasingly different sets of tissue-specific products. It is hoped that the isolation of embryonic neural-specific genes will allow the further analysis of these phenomena in the near future.

Molecular Genetics of fs(1)h, a Maternal Effect Homeotic Gene in Drosophila

Homeotic genes have attracted much attention because they appear to encode factors that are involved in the specification of segment identity in the fly. A classical example of a homeotic mutation is one that yields legs emerging from the head in place of antennae. There is much evidence to support the notion that homeotic genes control entire pathways of tissue differentiation, and thus constitute master control loci in development. The homeotic loci studied in many laboratories have exclusively or predominantly zygotic functions, i.e., the genotype of an individual determines its phenotype. However, much work over many years has suggested that there are factors stored in the oocyte that control subsequent development of the embryo; genes encoding such factors would constitute maternal-effect loci with profound effects on development. The locus studied by Igor Dawid and his colleagues, fs(1)h, has such effects: It encodes a product or products which is stored in the egg and is required for embryogenesis. Under certain conditions, mutant females are sterile, i.e., they lay eggs that die as early embryos, but under different conditions they produce progeny that exhibit transformation of body parts similar to those seen in bithorax mutations. These transformations are greatly enhanced in progeny flies that also carry mutations or hypodosage of other homeotic loci, especially of the trithorax (trx) gene. A molecular study of fs(1)h is therefore a promising avenue to understanding certain aspects of developmental regulation through oocyte factors.

The genome region containing fs(1)h has been cloned previously by chromosomal walking. Recently, transcription analysis demonstrated considerable complexity in the RNA products of this region. Two major ovarian RNAs, five larval RNAs, and one pupal RNA have been identified; they are generated from the same 20 kb-long region by alternate splicing, alternate 3' ends, and possibly alternate starting points. cDNA clones have been isolated and are being sequenced at present with the aim of predicting protein products for the locus. Fusion proteins between portions of fs(1)h and β-galactosidase have been generated and are being expressed in E. coli; the fusion proteins will be injected into rabbits for production of antibodies that should aid in the analysis of the natural products of the fs(1)h gene.

Integration of Macromolecular Synthesis in E. coli

Michael Cashel and his colleagues have continued their studies on global control of metabolism in E. coli, an interest similar to that of Hinnebusch in yeast. This term implies a regulatory system which allows the cell to respond to external conditions, e.g., nutritional deprivation, with adjustments in overall gene expression and metabolism. Different types of mechanisms are known that mediate the control of many genes in such responses; a major mechanism, and the one studied by Cashel's group, involves the unusual nucleotide ppGpp. High levels of ppGpp in the cell are correlated with inhibition of transcription of rRNA, tRNA and other genes encoding components of the protein synthesis machinery, but with stimulation of expression of amino acid biosynthetic enzymes and heat shock proteins, as well as enhanced proteolysis and accuracy of translation. The phenomena studied in detail in this laboratory concern inhibition of rRNA synthesis and stimulation of histidine operon expression.

An important tool in this work has been the development of bacterial strains in which the level of ppGpp can be varied experimentally without changing the richness of the growth media. This has become possible through the study of the spoT gene which encodes a 3'-pyrophosphatase that accounts for the major pathway of degradation of ppGpp. The spoT gene has been studied in its own right, yielding its sequence and allowing the construction of deletions and insertions that will be used in studying the functions of this gene further. The use of spoT mutations in changing in vivo ppGpp concentrations at will resulted in the isolation of rRNA promoter mutations that are insensitive to ppGpp; these mutations will be most helpful in future work. Further, it could be shown that the activity of the rRNA gene promoter P1 correlates inversely with ppGpp concentrations over the entire physiological range, suggesting that ppGpp interacts directly with the promoter.

Detailed studies have continued on several unusual features of rRNA operons, including dual promoters, antitermination, and dual terminators that apparently can stop antiterminated transcripts (supertermination). Novel results in this area includes the finding of an antitermination region just upstream of the 23S RNA gene, in an analogous position to the previously detected region upstream of the 16S RNA gene. The question of why two antitermination regions occur is being studied.

Enzymes Involved in RNA Processing

Robert Crouch directs a program with the aim of analyzing certain aspects of RNA processing. It has been clear for some time that all RNA molecules are modified in various ways after they are synthesized and before they function: the polymerization of nucleotides into an RNA chain is but the initial step in its production, and subsequent events are crucial in generating a functional entity. Certain RNAs act as primers in replication, and enzymatic mechanisms must exist that remove these primers after they have completed their function. Crouch and his colleagues have focused on an enzyme, RNase H, that appears to be involved in this event. RNase H, an enzyme that digests the RNA strand of RNA/DNA hybrids, occurs in all cells from bacteria to mammals, but its function is not fully understood.

The RNase H gene has been cloned from E. coli, Salmonella, and yeast, and the sequences of these genes have been determined. Comparisons of these species and mutant forms of the E. coli enzyme have provided insights into enzyme structure. In yeast, three distinct RNase H proteins have been found. This fact may explain the finding that inactivation of the one RNase H gene that has been cloned does not lead to any obvious phenotype.

The second area of interest in this group concerns RNA processing in mammalian cells. A mutant line of BHK cells impaired in the formation of 28S rRNA due to a processing defect has been studied. Transfection of cDNA generated from normal mouse cells has resulted in the correction of the processing defect, suggesting that the wild type gene has been introduced into the mutant cell. This result should help in determining the nature of the function which is defective in the mutant line and through it, aid in analyzing the mechanism of rRNA processing.

Control Mechanisms in Bacteriophage Lambda

Robert Weisberg and his colleagues have continued their studies on mechanisms of recombination and gene expression of bacteriophage λ, a prototypical and elegant model for in vivo studies on the structure and funciton of DNA. An important component of regulation of gene expression is termination of transcription at specific sites in the DNA called terminators. Termination may be enhanced or suppressed by protein factors; work in the current year has provided evidence in support of the view that the mechanisms of termination enhancement and suppression are closely related. This conclusion stems from work with a protein encoded by the λ-related phage HK022, called Nun, which promotes termination or antitermination, depending on the gene affected. Nun appears to act as an antitermination factor on its own genome, HK022; on the related phage λ, the same protein promotes termination. In the latter action, it requires the same host cell factors and sites that are required for antitermination promoted by the λ N protein. These findings suggest a fundamental unity in the mechanisms of termination and antitermination.

A major interest of this research group has been the mechanism of recombination, specifically during integration and excision of phage λ from the bacterial chromosome. Site-specific recombination promoted by the int protein occurs at attachment sites that contain a central core of seven homologous nucleotides. Mismatched attachment sites do not recombine efficiently. Recent work suggests that even mismatched sites form the recombinational intermediate called a Holiday structure, but that they are resolved only to parental rather than recombinant molecules. In the interaction between homologous sites, branch migration across the region of homology allows resolution towards the recombinant at high frequency; facilitation of branch migration may be the key function of the homologous core region of attachment sites.

Molecular Aspects of Replication of Enveloped Animal RNA Viruses

Judith Levin's group is concerned with the mechanism of replication of enveloped RNA viruses, specifically murine leukemia viruses (MuLV). Studies on the RNA tumor viruses are of particular interest since these viruses establish a chronic infection in the host cell by integrating viral genetic information into the host chromosome, often disrupting normal gene function. A detailed understanding of the steps involved in viral replication may permit the design

of agents which would limit the oncogenic potential of these viruses. Moreover, evidence is accumulating that a number of eukaryotic genetic elements have been created by transfer of information from RNA to DNA during the development of the eukaryotic genome.

Current interest in this program is focused on the process of reverse transcription in an effort to correlate genetic structure with enzymatic function. Portions of the pol gene have been expressed in E. coli and the products were used to generate antibodies specific for polymerase and endonuclease. These reagents have proved valuable in dissecting the relationships between these gene products. Recently, studies with an antiserum directed against endonuclease alone have led to the observation that the viral reverse transcriptase and endonuclease can be associated as a complex involving both non-covalent and disulfide bonds. The exact nature of this association within the virion is under investigation. The sera are also being used to define the defect in a viral mutant with an in-frame deletion in the endonuclease coding region. In addition, a clone expressing only reverse transcriptase sequences has been used to make a monospecific anti-polymerase serum and to provide a source of highly active enzyme. Large scale preparations of bacterial extracts of this clone have now been extensively purified by standard biochemical procedures, and characterization of the purified bacterially expressed reverse transcriptase is in progress.

Laboratory of Molecular Genetics

Chief, *Dr. Igor B. Dawid, Ph.D.

Senior Staff

*Michael C. Cashel, M.D., Ph.D.
Robert J. Crouch, Ph.D.
Judith G. Levin, Ph.D.
*Robert A. Weisberg, Ph.D.
*Heinrich J. Westphal, M.D.

Fellows

Shaw C. Chen, M.D., Ph.D.
Gary Fabian, Ph.D.
Ann E. Griep, Ph.D.
Susan Ann Haynes, Ph.D.
Alan G. Hinnebusch, Ph.D.
Kathleen Ann Mahon, Ph.D.
Mark Mangano, M.D.
Kenneth Rudd, Ph.D.
Thomas D. Sargent, Ph.D.
Sharon Zemel, M.D.

Visiting Program

Chikh Bengra, Ph.D.
Naina Bhatia-Dey, M.D.
Anup K. Dey, Ph.D.
Francoise Forquignon, Ph.D.

---

* Denotes Section Heads

Satoshi Harashima, Ph.D.
Mitsuhiro Itaya, Ph.D.
Milan Jamrich, Ph.D.
Erzsebet Jonas, Ph.D.
Masashi Kawaichi, M.D., Ph.D.
Jaspal S. Khillan, Ph.D.
Bernd Krippl, Ph.D.
Laurence Lempereur, Ph.D.
Seiji Miyatani, Ph.D.
Peter Muller-Koppel, Ph.D.
Takafumi Nakamura, Ph.D.
Jacques Oberto, Ph.D.
Christopher Paddon, Ph.D.
Frederic Rosa, Ph.D.
Edoardo G. Sarubbi, Ph.D.
Ezra Yagil, Ph.D.

Expert

George Michaels, Ph.D.

Guest Researchers

Sunil Chatterjee, Ph.D.
Adolf Graessmann, Ph.D.
Ernest Hannig, Ph.D.
Der-Hwa Huang, Ph.D.
Susan LaFlamme, Ph.D.
Ruth Miskin, Ph.D.
Klaus Richter, Ph.D.
Norma Williams, Ph.D.

Bibliography

Chambers C, Dutta SK, Crouch RJ. Neurospora crassa ribosomal DNA: sequence of internal transcribed spacer and comparison of neurospora crassa, restriction nuclease mapping. Gene 1986;44:159-64.

Crouch RJ, Bachellerie JP. Ribosomal RNA processing sites. In: Dutta SK, ed. DNA systematics. Boca Raton: CRC Press, 1986:47-80.

Dawid IB, Haynes SR, Jamrich M, Jonas E, Miyatani S, Sargent TD, Winkles JA. Gene expression in Xenopus embryogenesis. J Embryol Exp Morphol 1985;89:113-24.

Dawid IB, Rebbert ML. Expression of ribosomal insertion in Drosophila sensitivity to intercalating drugs. Nucleic Acids Res 1986;14:1267-77.

Dawid IB, Sargent TD. Molecular embryology in amphibians: new approaches to old questions. Trends in Genetics 1986;2:47-50.

Digan ME, Haynes SR, Mozer BA, Dawid IB, Forquignon F, Gans M. Genetic and molecular analysis of fs(1)h, a maternal effect homeotic gene in Drosophila. Dev Biol 1986;114:161-9.

Hinnebusch AG. The general control of amino acid biosynthetic genes in the yeast Saccharomyces cerevisiae. CRC Crit Rev Biochem 1986;21:277-317.

Hinnebusch AG. Multiple levels of gene regulation in the control of amino acid biosynthesis in Saccharomyces cerevisiae. BioEssays 1986;5:57-62.

Hinnebusch AG. Translational control of the positive regulator of amino acid biosynthetic genes in yeast. In: Matthews BM, ed. Current communications in molecular biology: translational control. NY: Cold Spring Harbor Laboratory, 1986;169-72.

Hinnebusch AG, Lucchine G, Fink GR. A synthetic HI54 regulatory element confers general amino acid control on the cytochrome c gene (CYC1) of yeast. Proc Natl Acad Sci 1985;498-502.

Hu SC, Court DL, Zweig M, Levin JG. Murine leukemia virus pol gene products: analysis with antisera generated against reverse transcriptase and endonuclease fusion proteins expressed in Escherichia coli. J Virol 1986;60:267-74.

Jamrich M. Polytene chromasomes. In: Risley MS, ed. Chromasomes structure and function. New York: Van Nostrand Reinhold Company Inc. 1986;221-43.

Jamrich M, Sargent TD, Dawid IB. Altered morphogenesis and its effects on gene activity in Xenopus laevis embryos. Cold Spring Harbor Symp Quant Biol 1985;50:31-5.

Khillan JS, Schmidt A, Overbeek PA, de Crombrugghe B, Westphal H. Developmental and tissue specific expression directed by the alpha 2 type I collagen promoter in transgenic mice. Proc Natl Acad Sci 1986;83:725-9.

Krippl B, Andrisani O, Jones N, Westphal H, Rosenberg M, Ferguson, B. Adenovirus type 12 E1A protein expressed in Escherichia coli is functional upon transfer by microinjection or protoplast fusion into mammalian cells. J Virol 1986;59:420-7.

Krippl B, Ferguson B, Jones N, Rosenberg M, Westphal H. Mapping of functional domains in adenovirus E1A proteins. Proc Natl Acad Sci 1985;82:7480-84.

Krippl B, Ferguson B, Jones, N, Rosenberg M, Westphal H. Nuclear localization and gene activation in trans are mediated by separate domains of the adenovirus E1A. In: Botchan M, Grodzicker T, Sharp PA, eds. Cancer cells 4/DNA tumor viruses. NY: Cold Spring Harbor Laboratory, 1986;419-28.

Mattai IW, Zeller R, Carrasco A, Lienhard S, DeRobertis EM. Jamrich M. U snRNA gene families in Xenopus laevis. In: Maclean E, ed. Oxford surveys on eukaryotic Genes, Vol. 2. Oxford: Oxford University Press 1985;121-140.

Mueller PP, Hinnebusch AG. Multiple upstream AUG codons mediate translational control of GCN4. Cell 1986;45:201-7.

Nussinov R, Weisberg RA. Bacteriophage lambda int protein may recognize structural features of the attachment sites. J Biomolec Struc Dynamics 1986;3:1133-44.

Overbeek PA, Chepelinsky AB, Khillan JS, Piatigorsky J, Westphal H. Lens-specific expression and developmental regulation of the bacterial chloramphenicol acetyltransferase gene driven by the murine alpha A crystallin promoter in transgenic mice. Proc Natl Acad Sci 1985;82:7815-19.

Overbeek PA, Lai SP, Van Quill KR, Westphal H. Tissue specific expression in transgenic mice of a fused gene containing RSV terminal sequences. Science 1985;231:1574-77.

Sargent TD, Dawid IB, Jamrich M, Jonas E, Miyatani S, Winkles JA. Regulated activation of the zygotic genome in developing Xenopus laevis embryos. In: Gall JG, ed. Gametogenesis and the early embryo. New York: Alan R Liss Inc, 1985;333-45.

Sargent TD, Jamrich M, Dawid IB. Cell interactions and the control of gene activity during early development of Xenopus laevis. Dev Biol 1986;114:238-46.

Sharma RK. Biochemical control in adrenocortical carcinoma. In: Hollander VP, ed. Hormonal responsive tumors. New York: Academic Press, 1985;185-217.

Verma M, Ghosh B, Chambers C, Crouch RJ, Dutta SK. A ribosomal RNA gene clone isolated from Neurospora crassa wild strain 74A differs significantly from a similar clone isolated from its abnormal developmental slime mutant. Current Science of India 1985;54:1092-97.

Westphal H, Overbeek P, Khillan J, Chepelinsky A, Schmidt A, Mahon K, Bernstein K, Piatigorsky J, de Crombrugghe B. Promoter sequences of murine alpha 2(I) collagen or of avian sarcoma virus genes linked to the bacterial chloramphenicol acetyl transferase (CAT) gene direct tissue specific patterns of CAT expression in transgenic mice. Cold Spring Harbor Symp Quant Biol 1985;50:411-6.

Winkles JA, Sargent TD, Parry DAD, Jonas E, Dawid IB. A developmentally regulated cytokeratin gene in Xenopus laevis. Mol Cell Biol 1985;5:2575-81.

---

*These are published reports in which the first NIH author is an NICHD scientist. A more extensive 1986 bibliography, including collaborative reports and papers in press, is available upon request.

Laboratory of Developmental Pharmacology--
Daniel W. Nebert, M.D., Chief

This Laboratory studies the molecular mechanisms of expression of genes that encode drug-metabolizing enzymes. The clinical discipline devoted to the study of genetic differences in drug metabolism has been termed pharmacogenetics. Endogenous (constitutive) enzymes that metabolize drugs, as well as steroids, fatty acids, prostaglandins, leukotrienes, pheromones, thyroxine and biogenic amines, also metabolize the innumerable foreign compounds to which we are exposed. Hundreds of drugs and other chemicals are known to bind to cellular-receptors, and then to induce their own metabolism and/or to influence the metabolic fate of structurally-related compounds. In addition, steroids, prostaglandins, and small peptide hormones have been found to induce some of these enzymatic activities. The mechanisms surrounding the induction of the drug-metabolizing enzymes and the expression of these genes are of central importance not only to the investigation of carcinogenesis, mutagenesis, drug toxicity and teratogenesis, but to developmental and molecular biology in general.

The section led by D. Nebert is investigating the regulation and expression of genes encoding "Phase I" drug-metabolizing enzymes, most of which are P450 proteins, and certain "Phase II" drug-metabolizing enzymes. (Phase I generally refers to oxygenation or other initial metabolism, and Phase II to conjugation or other detoxification routes.) The P450 gene superfamily is presently known to comprise at least eight gene families, and a protein encoded by any one of these eight families is no more than 36% similar to a protein encoded by any of the other seven families.

Genes of the P450-I family are inducible by combustion products such as benzpyrene and tetrachlorodibenzo-p-dioxin (TCDD, "dioxin"). The P450-II gene family is the largest one, with at least five subfamilies (each about 50% similar to one another) that diverged from one another about 300-600 million years ago. Genes in the II-B and II-E subfamilies are phenobarbital- and ethanol-inducible, respectively; genes in the II-A, II-C and II-D subfamilies are expressed constitutively. Genes in the P450-III and P450-IV families are steroid- and clofibrate-inducible, respectively. Genes in the families XI and XII encode mitochondrial proteins that are responsible for steroid 11β-hydroxylation and cholesterol side-chain cleavage, respectively. Genes in families XVII and XXI encode enzymes for steroid 17 α-hydroxylation and 21-hydroxylation, respectively.

This section is currently studying the expression of the [Ah] gene battery, a group of five or more genes that are (i) under aromatic hydrocarbon (Ah) receptor regulation and (ii) sometimes activated during embryogenesis (differentiation) and tumorigenesis (dedifferentiation). These genes, which encode P_1450 and P_3450 (i.e., the two members of the P450-I gene family), NAD(P)H:menadione oxidoreductase ($NMOR_1$), glutathione transferase (GT_1) and UDP glucuronosyltransferase ($UDPGT_1$), have all been cloned and are now being characterized. There are striking differences in transcriptional regulatory mechanisms for activation of the P_1450 and P_3450 genes, as well as marked developmental and tissue-specific differences in gene expression. Upstream P_1450 regulatory sequences include (i) the TATA box, (ii) a TCDD-responsive element (about 1000 bp upstream from the mRNA cap site) that spans more than 200 bp and includes one or more enhancers of constitutive gene expression, and

(iii) a negative control element (between 400 and 800 bp upstream from the cap site) that participates in (iv) a negative autoregulatory loop. It appears that a repressor of constitutive P_1450 gene transcription requires activation by a P_1450-mediated metabolite, and that this repressor regulates constitutive transcription of the $NMOR_1$, GT_1 and $UDPGT_1$ genes as well. Genes for the Ah receptor as well as the putative repressor are also being cloned and characterized.

Projects in this section are devoted to: (1) fundamental molecular biology and genetics, (2)studies on the evolution of the P450 genes and their regulatory regions, including utilization of the methods of chromosomal walking and mapping, and (3) applications of potential clinical importance. Experimental systems include the use of inbred mouse strains, transgenic mice, recombinant DNA technology, and somatic cell genetics. As an example of an application with potential clinical significance, the human P_1450 and P_3450 genes and their flanking regions have been cloned and sequenced, and localized near the MPI gene on chromosome 15. Evidence has been presented to suggest that human P_1450 and P_3450 genes, similar to their orthologues in laboratory animals, are important in the activation of inert chemical procarcinogens, promutagens and proteratogens to active metabolites. Restriction fragment length polymorphisms (RFLPs) have been found, and families with high and low cancer incidence are being studied. In the future it should be possible to correlate RFLP patterns of these genes with human disease. Such tests would greatly facilitate the evaluation of the cancer and toxicity risk in individuals exposed to specific foreign chemicals. These assays would aid the individual, employer and physician in decisions regarding life style, cigarette smoking, workplace, and the use of prescription drugs.

The section directed by H. J. Eisen compares the mechanism of action of the glucocorticoid receptor with that of the Ah receptor. Recently, it has been appreciated that the glucocorticoid and other steroid hormone receptors comprise a supergene family which includes the erb-A oncogene. Since the Ah receptor also binds hydrophobic ligands and interacts with DNA, it is possible that the Ah receptor is also a member of this supergene family.

This group has focused on the development of immunochemical and recombinant DNA probes for isolating and characterizing the Ah receptor protein and gene. Like steroid receptors, the Ah receptor is present at extremely low concentrations in cells; nonetheless, these workers have attempted to produce polyclonal antisera to receptor preparations which have been purified by affinity chromatography and HPLC methods. Interestingly, the Ah receptor copurifies with a group of mouse "heat shock" proteins which have the capacity also to bind hydrophobic ligands such as free fatty acids. Further studies of the structural and functional relationship of the Ah receptor to these heat shock proteins are in progress. Identification of the cDNA encoding the Ah receptor will require demonstration of the expressed functional gene product. Accordingly, in collaboration with the Section on Pharmacogenetics and Molecular Teratology, expression vectors in the yeast S.cerevisiae are being developed. It is hoped that such yeast expression systems will be useful for the ultimate characterization of the Ah receptor cDNA.

In other work, carried out in collaboration with Dr. S. Simons, the affinity-labeling steroid [^{3}H]dexamethasone 21-mesylate has been found to bind covalently to a single cysteine residue near the carboxy terminus of the glucocorticoid

receptor. These results identify rigorously for the first time the ligand-binding "pocket" of a steroid hormone receptor.

The unit led by P. I. Mackenzie studies the regulation and expression of several subfamilies of the rat UDP glucuronosyltransferase (UDPGT) gene family. The function of the "Phase II" UDPGT enzymes is to conjugate oxygenated (or N-or S-containing) metabolites with glucuronic acid, thereby rendering the glucuronide conjugate extremely hydrophilic, and thus detoxified and readily excreted. It follows logically that if a P450 enzyme oxygenates a hydrophobic drug or other chemical (Phase I metabolism), and UDPGT conjugates the oxygenated intermediate (Phase II metabolism), the two gene systems might be under some sort of coordinate regulation. Interestingly, UDPGT enzymes are similar to P450 enzymes in that (i) some genes are expressed constitutively and (ii) others are inducible by (a) combustion products such as benzpyrene and TCDD, (b) phenobarbital, (c) steroids or (d) peroxisome proliferators such as clofibrate.

The isolation and sequencing of seven cDNA clones has demonstrated the existence of at least four different forms of transferase belonging to two gene subfamilies. One clone, pUDPGT$_r$-2, encodes a phenobarbital-inducible form of transferase which glucuronidates 4-methylumbelliferone and the 17-OH position of testosterone and dihydrotestosterone; this was demonstrated by transfection of an expression vector containing the rat cDNA into monkey kidney fibroblast (COS) cells. This technique also demonstrated that a second clone, pUDPGT$_r$-4, the mRNA of which is not induced by phenobarbital or 3-methylcholanthrene, encodes a form of transferase that glucuronidates the 3-OH position of androsterone and etiocholanolone. The substrate specificity of other transferase forms is currently under investigation. Sequence studies have also shown that the transferases encoded by the three cDNAs contain signal peptide and membrane-anchoring regions and potential asparagine-linked glycosylation sites. In vitro translation studies indicate that the signal sequence is cleaved during insertion into the endoplasmic reticulum, and glycosylation occurs subsequently. Genomic clones xorresponding to UDPGT$_r$-4 have been isolated and are being sequenced. Genomic and cDNA clones will be utilized to study the regulation of each form as a function of age, tissue distribution and administration of prototypic inducers.

The section led by I. S. Owens examines the UDP glucuronosyltransferase gene family in the mouse and human. One cDNA clone has been isolated by screening a mouse cDNA-containing λgt11 expression library with P. Mackenzie's rat cDNA clone, UDPGT$_r$-2. By sequence analysis, this clone, UDPGT$_m$-1, encodes the complete protein sequence (530 amino acids) of a mouse cDNA that is highly homologous to UDPGT$_r$-2 and hybridizes with two phenobarbital-inducible mRNAs (2.0 and 2.2 kb) that are the result of alternative polyadenylation splicing sites. Both of these mRNAs are also induced by clofibrate (as is bilirubin UDP glucuronosyltransferase). UDPGT$_m$-1 was used to screen a human cDNA library; a human cDNA clone (UDPGT$_h$-1), which hybridizes to an mRNA of 2.5 kb, was isolated and is being sequenced. From a 3-methylcholanthrene-induced mouse cDNA-containing λgt11 expression library, an antibody to 3-methylcholanthrene-induced UDPGT was used to isolate UDPGT$_m$-2. This mouse cDNA insert is 2.4 kb in size and appears to be regulated by the Ah receptor.

Because of the clinical importance of diseases of defective bilirubin conjugation, $UDPGT_h$-1 may prove to be a valuable probe in subsequent studies on genetic screening and diagnosis. If $UDPGT_m$-2 is a member of the [Ah] gene battery, this gene may be of importance in fundamental molecular biologic studies of gene expression, as well as the detoxification of chemical carcinogens, mutagens and teratogens.

Laboratory of Developmental Pharmacology

Chief, *Daniel W. Nebert, M.D.

Senior Staff

*Howard J. Eisen, M.D.
*Ida S. Owens, Ph.D.

Fellows

Cynthia A. Edwards, Ph.D.
David M. Koeller, M.D.

Visiting Program

Sk. Jaharul Haque, Ph.D.
Kiyoko Ikeya, Ph.D.
Anil K. Jaiswal, Ph.D.
Jong-Youn Lee, Ph.D.
Peter I. Mackenzie, Ph.D.
Osamu Michioka, Ph.D.
John A. Robertson, Ph.D.
Yhun Y. Sheen, Ph.D.

Experts

Alvaro Puga, Ph.D.

Guest Researchers

John Jones, Ph.D.
Roland A. Owens, Ph.D.
Vesna Rapic, D.Sc.
Baisakhi Raychaudhuri, Ph.D.

Bibliography

Bigelow SW, Nebert DW. The murine aromatic hydrocarbon responsiveness locus: a comparison of receptor levels and several inducible enzyme activities among recombinant inbred lines. J Biochem Toxicol 1986;1:1-14.

Chen YT, Nebert DW. Recombinant DNA approaches to human pharmacogenetic disorders. In: Wu M, Nebert DW, eds. Genes and disease. Beijing: Science Press, 1986;59-79.

Eisen HJ. Induction of hepatic P-450 isozymes: evidence for specific receptors. In: Ortiz de Montellano, PR, ed. Cytochrome P-450: structure, mechanism and biochemistry. New York: Plenum Publishing Corp, 1986;315-44.

Gonzalez FJ, Nebert DW. Autoregulation plus upstream positive and negative control regions associated with transcriptional activation of the mouse P_1450 gene. Nucleic Acids Res 1985;13:7269-88.

Jaiswal AK, Nebert DW. Two RFLPs associated with the human P_1450 gene linked to the MPI locus on chromosome 15. Nucleic Acids Res 1986;14:4376.

Jaiswal AK, Nebert DW, Gonzalez FJ. Human P_3450: cDNA and complete amino acid sequence. Nucleic Acids Res 1986;14:6773-74.

Kimura S, Gonzalez FJ, Nebert DW. Tissue-specific expression of the mouse dioxin-inducible P_1450 and P_3450 genes: differential transcriptional activation and mRNA stability in liver and extrahepatic tissues. Mol Cell Biol 1986;6:1471-77.

Kimura S, Nebert DW. cDNA and complete amino acid sequence of mouse P_2450: allelic variant of mouse P_3450 gene. Nucleic Acids Res 1986;14:6765-66.

Kouri RE, McLemore T, Jaiswal AK, Nebert DW. Current cellular assays for measuring clinical drug metabolizing capacity--impact of new molecular biologic techniques. In: Kalow W, Goedde HW, Agarwal DP, eds. Ethnic differences in reactions to drugs and xenobiotics. New York: Alan R Liss, Inc, 1986;453-69.

Mackenzie PI. Rat liver UDP-glucuronosyltransferase: cDNA sequence and expression of a form glucuronidating 3-hydroxyandrogens. J Biol Chem 1986;261:14112-7.

Mackenzie PI. Rat liver UDP-glucuronosyltransferase: sequence and expression of a cDNA encoding a phenobarbital-inducible form. J Biol Chem 1986; 261:6119-25.

Nebert DW. P450 genes and their regulation. Trends Pharmacol Sci [Meeting Report] 1985;6:270-73.

Neuhold LA, Gonzalez FJ, Jaiswal AK, Nebert DW. Dioxin-inducible enhancer region upstream from the mouse P_1450 gene and interaction with a heterologous SV40 promoter. DNA 1986;5:403-11.

Peterson TC, Gonzalez FJ, Nebert DW. Methylation differences in the murine P_1-450 and P_3-450 genes in wild-type and mutant hepatoma cell cultures. Biochem Pharmacol 1986;35:2107-14.

Tuteja N, Gonzalez FJ, Nebert DW. An example of P-450 catalytic activities not correlated with corresponding P-450 mRNA concentrations. Biochem Pharmacol 1986;35:718-20.

Laboratory of Neurochemistry and Neuroimmunology--
Harold Gainer, Ph.D., Chief

This Laboratory is concerned with the development, functional organization and interactions between three major integrative systems in the body - the central nervous system, the endocrine system, and the immune system. The approach of the Laboratory is cell biological in nature, and hence utilizes a wide variety of techniques and concepts from a number of disciplines, e.g., physiology, biochemistry, morphology, immunology, and molecular biology. In particular, these investigators study the various secretory peptides, intracellular membrane systems, and cytoskeletal proteins which are found in these organ systems and which are essential to their functions (i.e., peptide biosynthesis and regulation, neuronal morphology and function, etc.). A special emphasis is placed on the study of the cellular development of these organ systems.

The Section on Functional Neurochemistry, led by H. Gainer, focuses on the large number of neuropeptides which have been identified as chemical transmitters of information in the nervous system. These known peptides (and presumably those still to be discovered) act as conventional neurotransmitters in a synaptic, paracrine, or autocrine fashion, as well as in neuroendocrine systems. In addition to the well-known hypophysiotrophic regulatory peptide hormones (e.g., CRF, LHRH, and TRH), neuropeptides are involved in a wide variety of other CNS functions (e.g., pain, blood pressure control,and memory). This Section's goal is to study the cell biology of peptidergic neurons in the context of their regulatory functions in the nervous system. In particular,the lab specifically studies the expression of neuropeptides during CNS development and their impact on the development of organismic functions and morphology.

The studies of this group have been focused on two specific peptidergic systems in the hypothalamus, the oxytocinergic and vasopressinergic neuronal systems, and the LHRH neuronal system. The choice was made to concentrate on these systems because the neurons that constitute them represent excellent models of peptidergic neurons in the nervous system. The hypothalamo-neurohypophysial system is studied because the neurons that constitute it (i.e., the oxytocin and vasopressin magnocellular neurons) are prototypic of peptidergic neurons in the central nervous system. These neurons populate two defined topographic sites in the brain (the paraventricular and supraoptic nuclei), and have a specific axonal pathway and termination site, all of which are accessible to experimental manipulation *in vivo* by stereotaxic, morphological, and biochemical-pharmacological techniques. Access to all three of the critical neuronal structures, i.e., the cell body, the axon, and the terminal--in the hypothalamus, median eminence, and posterior pituitary, respectively--permits a cell biological analysis of this system. The cell bodies in the hypothalamus are responsible for the biosynthesis of the vasopressin (AVP) and oxytocin (OT) prohormones and peptides. In addition to examining these neurons in the mammal (rat and mouse), these investigators extended these studies to the neurons that synthesize the evolutionarily related vasotocin (AVT) and mesotocin (MT) peptides in the frog (*Xenopus laevis*). A second peptidergic system under study in the laboratory is the LHRH system in the rat. The LHRH neurons found in the hypothalamus project principally to the median eminence. The secretion of LHRH into the portal blood system regulates the development and function of the reproductive system. In addition to being the objects of cell biological studies, the embryonic development of these two neuronal systems is also currently under intensive study. A third peptidergic neuronal system found in the hypo-

thalamus is under study as well, i.e., the corticotropin releasing factor (CRF) system. This system projects to the external zone of the median eminence, where the secretion of CRF into the portal system causes the release of ACTH from corticotropes in the anterior pituitary. The section is particularly focusing on the co-existence of AVP in the CRF neurons, and the modulation of AVP biosynthesis and release during adrenalectomy and stress.

In addition to the above studies on peptidergic neurons, the Section has embarked on an analysis of macromolecular involvement in neuronal structure and recognition. Three model systems are being used for this purpose; the Xenopus developing nervous system, the squid giant axon, and the mouse hypothalamo-neurohypophysial system. Each of these systems makes a unique contribution to the study of neuronal structure and development (see below). The progress in each of the above projects will be described below in relation to the individuals who are the principal experimenters. The ability to make monoclonal antibodies (Mabs) against small quantities of antigens continues to be an important aim of the laboratory. S. House, a technician in the laboratory, has been studying how to optimize in vitro immunization protocols for this purpose. She has done extensive analyses of the efficacy of a variety of conditioning media (i.e., from mouse thymus, cow thymus, and EL-4 cells) to promote effective in vitro immunization. At present she is employing these techniques, in collaboration with the Unit on Neurosecretory Systems, to produce Mabs against calcium binding proteins (e.g., calsequestrin). Preliminary indications are that more than 21 producing clones are being harvested by these procedures. S. Key, another technician in the Section, is developing methods to use these Mabs and antisera in immunocytochemical procedures at the light and electron microscopic levels. These procedures, which include pre- and post-embedding staining, silver intensification, staining of cultured tissue, whole mounts, vibratome sections, etc., are ultimately used by all members of the Section in their research. At present, Ms. Key has successfully mastered these techniques and is educating various members in the laboratory on their use.

Progress in the analysis of the peptidergic neuronal system can be described as follows: M. H. Whitnall has extensively studied the response of the CRF system to adrenalectomy. He has demonstrated that: 1) the CRF neurons are composed (in normal rats) of two populations, one which contains AVP in co-existence with CRF in its secretory vesicles (50%) and one which only contains CRF (50%); 2) This co-existence phenomenon has been demonstrated by using three antibodies against the three components of the AVP prohormone (AVP, NP-VP, and the glycopeptide), with the findings raising the ultrastructural analysis of co-existence to a new level of comprehensiveness; 3) He has shown that following adrenalectomy there is only one population, i.e., the co-existent one, thereby demonstrating a neuronal plasticity relative to function in this system. These studies were done by serial section immunocytochemistry and quantitative analysis. M. Altstein has studied the development of the AVP and OT neuronal system by quantitative RIA methods, and has shown that AVP precedes OT expression by 6 days in embryonic development, and that OT expression is a post-natal phenomenon. In addition, she has evaluated the cause of this differential development and found that both AVP and OT prohormones are expressed concurrently but that the OT processing is delayed. Altstein has also discovered that in vivo, the first processing endopeptidase cleaves on the carboxyl-side of the Arg in the Lys-Arg signal within the OT-prohormone. K. Conway has analyzed the distribution of 4000 AVP and MT neurons in the adult Xenopus brain, and has analyzed the crossreactivities of mammalian NP Mabs in these neurons. He has discovered

a fascinating epitope switch in the frog NP versus the rat NP, which could be explained by a switch in exon C in the mammalian versus the frog gene. Pursuit of his main objective, to examine the lineages of the AVT and MT neurons in Xenopus, is now in progress. S. Wray has examined LHRH neuron development in the rat from the neonate (2 days pn) to adulthood. Her observations are that smooth LHRH cells are transformed to irregular LHRH cells during puberty, and that this occurs independently of gonadal steroid feedback. Current efforts are being directed at determining whether specific synaptic inputs to the LHRH cells are modified during this dramatic morphological change. This involves the use of double-label E.M. immunocytochemistry. In addition, Wray has developed an in vitro organotypic culture of the LHRH system, and has succeeded in growing substantial numbers of LHRH neurons for as long as one month in vitro. Of even greater significance, she has been able to co-culture brain stem, hypothalamus, and pituitary successfully so as to allow a study of these three-dimensional components in the brain in a two-dimensional space. She is currently studying trophic relations in these co-cultures.

Progress in the neuronal structure program is as follows: B. Szaro has completed his analysis of Mabs against the Xenopus neuronal cytoskeleton, and is currently using these characterized Mabs to evaluate the expression of cytoskeleton elements during nervous system development. Some of these brain specific Mabs (e.g., MAP-1,and neurofilaments) are recognizing specific cells and fibers in the nervous system as early as stage 33 in development, and efforts are currently underway to move to the neural plate stage. L. Charnas is currently screening a Xenopus brain cDNA library with intermediate filament probes, and several promising clones are under study. Both of these approaches (molecular and immunological) are directed at understanding the development of neuronal processes (e.g., axons and dendrites) in Xenopus.

H. Gainer's studies in the squid giant axon system on neurofilament structure and function continue to support the hypothesis that the genesis of neurofilament structure is determined topographically in the neuron, i.e., biosynthesis and assembly in the cell body, phosphorylation in the axon, and proteolysis in the terminal. This topographic organization of regulation of post-translational modification is currently under study with regard to mechanism.

A new program on cellular recognition in the nervous system, using the hypothalamo-neurophysial system, has been initiated. Y. Hara is currently attempting to isolate the AVP and OT genes of the mouse from a mouse genomic library, using human AVP and OT gene probes. The aim of this is to isolate the mouse 5'-flanking (promoter) sequences, so as to use these promoter regions in the transgenic mouse model. Given AVP or OT promoters linked to other structural genes (e.g., class I MHC, and NCAM), one can theoretically study the impact of expression of virtually any macromolecules on AVP and OT neuronal development. Finally, A. Nieburgs is beginning a project on the relationship of posterior pituitary astrocytes (i.e., pituicytes, glia) on nerve terminal development in the posterior pituitary.

The research goal of the Section on Cellular Neurobiology, led by Y. Peng Loh, is to study brain and pituitary peptides which are involved in intercellular neurocommunication and fetal development. The emphasis has been on the ACTH-endorphin/ α-MSH family of peptides. Endorphin is an opiate peptide that is

found in brain, pituitary and placenta. α-MSH is present in brain and pituitary, but in humans it is present in the pituitary only during pregnancy and in the fetus. This peptide has been implicated to have an effect on fetal growth and development. ACTH is a pituitary peptide which is traditionally known to stimulate steroidogenesis. However, it is also found in the brain. All of these peptides have been shown to have central nervous system effects and are thought to act as neurotransmitters and neuromodulators. The major focus has been to continue to study the enzymology and regulation of biosynthesis, packaging and secretion of this family of peptides. Within the past year two interrelated projects have been pursued.

The ACTH, α-MSH and endorphin peptides are synthesized in the intermediate lobe of the pituitary from a common, glycoprotein prohormone (pro-opiomelanocortin, POMC) of about 32,000 daltons in size. This group has assayed for several enzymes involved in the processing of this prohormone. These include a carboxypeptidase B-like enzyme, an aminopeptidase B-like enzyme, and a paired basic residue-specific prohormone converting enzyme (PCE). This latter enzyme has been purified to apparent homogeneity from secretory vesicles of the bovine pituitary intermediate lobe and neural lobe by Drs. Parish, Tuteja and Loh. PCE from both lobes appear to have very similar characteristics and are likely to be the same enzyme. PCE is a glycoprotein, has a molecular weight of ~70,000 daltons, and cleaves several precursors (POMC, pro-vasopressin, pro-insulin and pro-enkephalin) at paired basic residues to yield products seen in the tissues that synthesize these prohormones or neuropeptide precursors. The enzyme has a pH optimum of 4.0-4.5, consistent with intravesicular pH. Recently, using a simpler substrate, β-lipotropin (β-LPH) (the C-terminal part of POMC), Loh et al. determined the K_m and V_{max} of the cleavage of Lys_{37}-Lys_{38} and Lys_{57}--Arg_{58} of β-LPH to be 1.9 μM and 4.6 nmol/μg protein/h, and 2.5 μM and 9.1 nmol/μg protein/h, respectively. Inhibitor studies have shown that PCE is inhibited by two aspartyl protease inhibitors, pepstatin A and diazoacetyl-norleucine methyl ester, but not by thiol or serine protease inhibitors. Thus, PCE is the first aspartyl protease isolated in mammalian cells that has specificity towards basic residues. Further evidence that PCE is a physiologically significant enzyme in prohormone processing comes from recent studies showing that the enzyme is secreted from dissociated bovine intermediate lobe cells together with the hormone, α-MSH, and that the release of both is inhibited by dopamine. Finally, these workers have demonstrated that POMC processing is inhibited by peptstatin A in intact mouse neurointermediate lobes. This latter result shows that PCE has fulfilled the most stringent criteria for the identification of a physiologically relevant prohormone processing enzyme, as set forth by Docherty and Steiner (Ann. Rev. Physiol., 1982), i.e., an inhibitor of the putative enzyme must interfere with the precursor processing in the intact cells. PCE is the first enzyme demonstrated to process intact prohormones, and is of potential commercial value for the production of peptide hormones and neuropeptides when used for the limited cleavage of precursors synthesized by bacteria which have been transfected with a vector carrying the prohormone cDNA sequence. Loh et al. haye also purified sufficient PCE for amino acid sequence analysis and their goal in the coming year is to clone the gene which encodes the enzyme.

N. Birch is making good progress in the purification of the amino peptidase B-like enzyme from pituitary secretory vesicles. He has shown that the enzyme cleaves an Arg at least 6-fold faster than a Lys at the N-terminal of a peptide. This result is exciting since it can explain the accumulation of high

levels of Lys-γ-MSH in the intermediate pituitary, but no other Arg-extended, POMC-derived peptides, except when the Arg is followed by a Pro, as in CLIP. In this case the aminopeptidase B-like enzyme will not cleave the Arg.

Progress has also been made in a study that concerns the regulation of synthesis of pro-opiomelanocortin (POMC) in the toad intermediate lobe (IL). Using organ cultured toad neurointermediate lobes, B. Myers has shown that dopamine effectively down-regulates the biosynthesis of POMC. The dopamine receptor in the toad intermediate lobe was pharmacologically characterized as being in the D2 category and negatively coupled to adenylate cyclase. The presence of dopamine in the culture medium resulted in a decrease in intracellular cAMP and POMC synthesis in the intermediate lobe. This decrease in POMC synthesis was prevented by the addition of 8-Bromo-cAMP to the medium. B. Myers has also examined the regulation of POMC synthesis at the transcriptional level during black and white background adaptation of the frog. Using a 48 mer synthetic probe to frog POMC and quantitative in situ hybridization technology, she has shown that the POMC mRNA level of the IL is greatly increased during black background adaptation.

This year, S. Elkabes has initiated a study on the regulation of POMC synthesis in the same intermediate and anterior pituitaries of mice that have been subjected to hyperosmotic stress by supplying the animals with 2% saline in place of drinking water. Salt loading for two days resulted in a 175% increase in POMC mRNA levels in the anterior pituitary. POMC synthesis and processing was also enhanced in this tissue. In contrast, POMC mRNA levels and synthesis decreased by 50% in the intermediate lobe. Neuroendocrinological components (such as CRF and vasopressin) which may regulate these changes in POMC synthesis are currently being studied.

The Unit on Neuronal Secretory Systems, under J. Russell, investigates the nerve terminal, a highly specialized region of the neuron separated from the neuronal soma by an axon, whose function is to release neurotransmitter substances when stimulated by an electrical signal carried by the axon. The nerve terminal plays the central role in the nervous system function that operates by signal transmission between cells, by means of secretion of neurotransmitters. Modulation of the quantity of the transmitter released at the terminal may form the basis for all central nervous system functions, including integration of information, long term information storage, and retrieval. Because of this complexity (cellular heterogeneity, and their complex organization), a basic understanding of the molecular mechanisms of nerve terminal function in the central nervous system is still lacking.

The program of this lab is focused on studying the biochemistry and physiology of the nerve terminal using the neurohypophysial neuroendocrine cells as the model system. The hypothalamo-neurohypophysial system represents a model of central nervous system neurons, because of the homogeneity and discrete localization of these cells, which are eminently accessible for experimentation. The nerve terminals of these neurons are discretely localized in the neurohypophysis, where they are accessible to experimental manipulations both in vivo and in vitro. These nerve terminals can be isolated from the neurohypophyses without contamination by the post-synaptic membrane, unlike nerve terminals from other regions in the central nervous system.

Studies on the elucidation of the functional organization of the nerve terminal form the central theme of the Unit on Neuronal Secretory Systems. A preparation of neurosecretosomes (nerve endings from the neurosecretory neurons) has been obtained. This preparation is highly enriched in nerve terminals and forms the basic experimental model. Four different aspects of nerve terminal function are under investigation. First, the mechanisms of Ca^{2+} ion homeostasis in the nerve terminal are being investigated both from the membrane transport standpoint and from the standpoint of binding to an intraterminal Ca^{2+} binding protein. Secondly, the mechanism of secretory regulation at the nerve terminals is being studied with a view to understanding the molecular mechanisms of facilitation, and to characterize the physiological role of other peptide receptors on the nerve terminals in the regulation of secretion. Thirdly, studies on the transport processes in neurosecretory vesicles continue. Experiments are underway to identify conclusively the proton pumping Mg^{2+} ATPase on neurosecretory vesicles and to compare its properties with the mitochrondrial proton translocator. The neurosecretory vesicles are also studied to elucidate the presence of ionic channels intrinsic to this membrane. This study is carried out in collaboration with G. Ehrenstein and S. Elis of the Laboratory of Biophysics, NINCDS. Finally, in collaboration with I. Forsythe of the Laboratory of Developmental Neurobiology, experiments are underway to characterize membrane ionic channels on the nerve terminals using patch clamp techniques.

C. Bondy is involved in studies on elucidating the cellular mechanisms involved in frequency-dependent facilitation of vasopressin secretion from isolated neurohypophyses. Preliminary experiments suggest that K^+ channels are important in the facilitation of secretion. Dr. Bondy also has developed an immobilized neurosecretosome model to study the kinetics of secretion from nerve terminals in response to depolarizing stimuli.

J. Garbern has undertaken on studies of Ca^{2+}-binding proteins present in nerve terminals. He has detected two high affinity Ca^{2+}-binding proteins which are unique to nerve terminals, and is in the process of purifying them for characterization.

Laboratory of Neurochemistry and Neuroimmunology

Chief, *Harold Gainer, Ph.D.

Senior Staff

*Y. Peng Loh, Ph.D.
James T. Russell, Ph.D.

Fellows

Carolyn A. Bondy, M.D.
Kevin Conway, Ph.D.
James Y. Garbern, M.D.
Ben G. Szaro, Ph.D.
Mark H. Whitnall, Ph.D.
Susan Wray, Ph.D.

Visiting Program

Miriam Altstein, Ph.D.
Nigel Birch, Ph.D.
Maria Castro, Ph.D.
Stella Elkabes, Ph.D.
Yoshinobu Hara, Ph.D.

Guest Researchers

Andra Nieburgs, Ph.D.
Hanna Parnas, Ph.D.

Bibliography

Castel M, Morris J, Ben-Barak Y, Timberg R, Sivan H, Gainer H. Ultrastructural localization of immunoreactive neurophysins using monoclonal antibodies and protein A-gold. J Histochem Cytochem 1986;33:1015-25.

Castel M, Morris JF, Whitnall MH, Sivan N. Improved visualization of the immunoreactive hypothalamo-neurohypophysial system by use of immunogold techniques. Cell Tissue Res 1986;243:193-204.

Gainer H, Wolfe S, Obaid AL, Salzberg BM. Action potentials and frequency dependent-secretion in the mouse neurohypophysis. Neuroendocrinology 1986;43:557-63.

Lang MA, Handler JS, Gainer, H. Neurohypophysial peptide potencies in cultured anuran epithelia (A-6). Amer J Physiol 1986;251:R77-81.

Lang MA, Muller J, Preston AS, Handler JS. Complete response to vasopressin requires epithelial organization in A6 cells in culture. Am J Physiol 1986;250:C138-45.

Loh, YP. Kinetic studies on the processing of beta$_h$-lipotropin by bovine pituitary intermediate lobe pro-opiomelancortin converting enzyme. J Biol Chem 1986;261:11949-55.

Parish DC, Tuteja R, Altstein M, Gainer H, Loh YP. Purification and characterization of a paired basic residue-specific prohormone-converting enzyme from bovine pituitary neural lobe secretory vesicles. J Biol Chem 1986;261:14392-7.

Salzberg BM, Obaid AL, Gainer H. Large and rapid changes in light scattering accompany secretion by nerve terminals in the mammalian neurohypophysis. J Gen Physiol 1985;86:395-411.

Salzberg BM, Obaid AL, Gainer H. Optical studies on ionic channels in intact vertebrate nerve terminals. In: Latore R, ed. Proceedings of UNESCO International School on Ionic Channels. New York: Plenum Press, 1986;141-62.

Salzberg BM, Obaid AL, Gainer H. Optical studies of excitation and secretion at vertebrate nerve terminals. In: DeWeer P, Salzberg BM, eds. Optical methods in cell physiology. New York: John Wiley and Sons, 1986;131-64.

Whitnall MH, Gainer H. Ultrastructural studies of peptide co-existence in corticotropin releasing factor and arginine vasopressin-containing neurons. In: Moody TW, ed. Neural and endocrine peptides. New York: Plenum Press, 1986;159-75.

Whitnall MH, Gainer H. Ultrastructural immunolocalization of vasopressin and neurophysin in neurosecretory cells of dehydrated rats. Brain Res 1985;361:400-4.

Laboratory of Comparative Ethology--
Stephen J. Suomi, Ph.D., Chief

The Laboratory of Comparative Ethology (LCE) carries out a broad program of research directed toward the study of behavioral development in humans and in nonhuman primates. The influence on developmental processes of both genetic and environmental factors, and their interactions, is explored in a comparative mammalian approach to determine the origins and evolution of various behavioral phenotypes. Longitudinal designs are employed in most developmental studies within the Laboratory, in order to address issues of ontogenic continuity versus change, and both behavioral and physiological measures--reflecting multiple levels of analysis--are collected concomitantly. A major emphasis is placed on characterizing and understanding normative patterns of biobehavioral development so that deviant patterns can then be readily recognized and their consequences evaluated with respect to established norms. Experimental results in nonhuman primates are correlated with the results of longitudinal studies of human infants and their families, as well as results obtained by various neuroscience techniques.

Comparative Behavioral Genetics: Primate and Human

These studies, led by Stephen Suomi, are concerned with various processes underlying biological and behavioral development in rhesus monkeys, and focus on interactions between genetic and environmental factors that affect the course of an individual's ontogeny. Parallel patterns are examined in humans. Two major longitudinal studies investigating the interaction of genetic and environmental factors in shaping individual rhesus monkey development were completed this year. In the first study, rhesus monkey infants selectively bred to be highly responsive to mild environmental challenges ("uptight"), were cross-fostered by multiparous females who differed with respect to their own reactivity to challenge and in their characteristic maternal "style" (nurturant versus punitive) as displayed toward previous offspring. The highly reactive infants were compared with control infants ("laid-back") cross-fostered by multiparous females with either of the two "styles" and levels of reactivity. Neonatal tests of reflex development, orienting capability, and temperament administered to these subjects during their first month of life revealed that the infants with high reactive pedigrees showed poorer orienting responses, were less mature with regard to motor activity, and displayed greater behavioral inhibition than did control infants. Differences in these one-month scores could not be attributed to either the level of reactivity or the characteristic maternal style of the foster mothers. The infants remained with their foster mothers throughout the first 6 months of life, and during this time there were no major differences between high reactive and control infants on standard measures of behavioral development. Small individual differences among infants during this period were best predicted by the maternal style of their foster mothers, in that infants of punitive foster mothers displayed higher levels of self-directed behavior than did infants with nurturant foster mothers; these differences beccame greater as the infants passed through the period of weaning. When the subjects were 6 months old, they were briefly separated from their foster mothers. During the period of separation, dramatic differences in response emerged between subjects of high reactive pedigree and their controls, independent of foster mother reactivity or maternal style: High reactive monkeys displayed higher levels of self-directed behavior, less locomotion and exploration (coping), greater passivity, and higher increases

(over preseparation levels) of plasma cortisol. When the subjects, now juveniles, were reunited with their foster mothers, major differences between high reactive subjects and controls again disappeared. During reunion periods the best predictor of small differences between subjects was the relative reactivity of the foster mothers, in that juveniles with "uptight" foster mothers displayed higher levels of disturbance behavior than did those with "laid-back" foster mothers. These differences emerged independent of the juveniles' reactivity. Subjects were permanently moved into groups containing agemates at 8 months of age and have remained in these peer groups to the present. Within these now stable groups, the high reactive juveniles were initially more aggressive than were controls, and they continued to show higher levels of clinging social contact than controls during the next 6 months. Those high reacting subjects who had been reared by nurturant foster mothers rose to the top of the group's dominance hierarchy. These monkeys will continue to be studied at least to early adulthood, but the findings to date clearly indicate that individual differences displayed during early development are the product of both genetic and environmental factors, and that to the degree that these factors can be specified,a rather precise prediction of developmental outcomes in various settings is possible.

Genetic-environmental interactions were further investigated in a second longitudinal study comparing nursery-reared versus mother-reared rhesus monkeys with respect to neonatal reflexes, orienting, and temperament assessments, as well as performance on tests of cognitive capabilities as juveniles. Surprisingly, the profiles of neonatal scores associated with optimal performance on the later cognitive tests were dramatically different for the mother-reared and nursery-reared subjects, in that mother-reared juveniles who as neonates had "easy" temperaments had the best performance within their rearing group on the cognitive tests, whereas those nursery-reared subjects who were "fussy"and highly aroused when assessed as neonates had the best 8-month performances within their rearing group. Thus, the same top scores on the cognitive test battery were achieved by individuals with very different neonatal temperaments when they grew up in very different rearing circumstances.

During the past year, a number of new assays were incorporated into ongoing longitudinal studies of rhesus monkeys differing in their behavioral phenotypes. A means for recording heartrate and monitoring vagal tone (a measure of sympathetic/parasympathetic balance) in free-ranging monkeys, using a miniaturized telemetry system, was implemented in a study comparing the responses of juvenile rhesus monkeys genetically differing in reactivity. Subjects were placed with an unfamiliar agemate in a laboratory playroom filled with toys for a 1-hour period, a paradigm adapted from research on human preschool children with different temperaments. In this situation, it was found that high reactive juveniles had higher heartrates upon entry into the playroom, and showed less decline in heartrate over the 1-hour session than did control subjects. The vagal tone measures are currently being analyzed.

Data from a study of the behavioral and physiological effects of imipramine treatment of adolescent rhesus monkeys under conditions of group versus individual housing were subjected to additional analyses in the past year. Notably, analysis of blood levels of imipramine and its metabolites revealed marked differences in imipramine turnover when subjects were housed together in familiar social groups, or individually housed for 4-day periods. In particular,

blood levels of imipramine were much lower, and levels of the metabolites much higher, when subjects were individually housed. These findings suggest different pathways for metabolizing imipramine under conditions of environmental challenge. A second study, involving acute rather than chronic administration of imipramine, has been completed and the data are currently under analysis.

An ongoing study of behavioral development and social organization was continued in a group of 13 year-old free-ranging rhesus monkeys who, despite being reared without mothers and with only minimal peer contact, have displayed species-normative behavioral repertoires (as have 2 generations of progeny). The data continue to verify the species-normative behavioral patterns in the adults and ontogenic changes in the infants, the characteristic social organization of the troop along matriarchal lines, the species-appropriate changes in social rank among troop members (most notably the drop in dominance and eventual peripheralization of adolescent males), and the successful maternal care displayed by second-generation females.

Finally, two major research projects involving developmental study of human infants were initiated. A pilot study of individual differences in behavioral and physiological responsiveness to mild environmental challenge was conducted in collaboration with Drs. K. Grossmann (University of Regensburg, West Germany) and Dr. S. Porges (University of Maryland). Ten-month-old infants were brought into an examining room by their mothers and after producing a saliva sample (to be assayed for cortisol) and being fitted with a nonobtrusive telemetry system for recording heartrate and vagal tone, the infants were put through a standard pediatric examination, after which a second saliva sample was obtained. Two months later each infant returned to the laboratory with his or her mother and after producing a saliva sample and being fitted with the heartrate telemetry system again, the infant was put through the Ainsworth Strange Situation Procedure (a standard experimental paradigm in the human mother-infant attachment research field), after which a second saliva sample was obtained. Both experimental sessions were videotaped for subsequent behavior analysis. The data from this study will delineate the effects of the two manipulations on saliva cortisol, heartrate, vagal tone, and behavior, permitting assessment of the stability of individual differences between the two situations. These findings will then be compared with data from parallel studies of individual differences in response to environmental challenge in rhesus monkey infants and juveniles, in which considerable stability over time and across situations has already been found.

The second set of human infant studies was carried out in collaboration with Drs. H. and M. Papousek of the Max Planck Institute of Psychiatry (Munich) and David Symmes, of the LCE. These studies focused on the phenomenon of "intuitive parenting," in which preverbal human infants elicit a distinctive pattern of communication from parents and other caretakers that includes speech ("baby-talk"), exaggerated facial expressions, and maintenance of eye-to-eye contact at a fixed distance. Such distinctive communication patterns are produced by parents automatically and usually without conscious awareness that they are interacting with the infant in a different fashion than they would with adults. In the main study for which data collection was completed this year, mothers whose native language is either English or Mandarin Chinese (a language characterized by extreme tonality) brought their 2-month-old infants into a laboratory playroom, and in that setting their normal interactions with their infants were videotaped over a half-hour period. When their infants were

4 months old, the mothers and their infants returned to the laboratory playroom for a second interaction session, which was also videotaped. The pitch patterns and contours of both mothers and infants were then subjected to microanalytic spectrographic analysis, using computer-aided array processing techniques. The maternal utterances have also been transcribed, translated (for Chinese speaking mothers), and coded for linguistic content, and microanalysis of changes in both the mothers' and infants' facial expression has begun. Additional cross-cultural comparisons, using existing videotapes of mother-infant interactions in West German, African pigmy, Yucatan Indian, and deaf American populations are also underway, with preliminary data indicating great generality of the phenomenon of intuitive parenting across all cultures sampled, but with some differences in the relative frequency and developmental course of the phenomenon from one culture to another. Finally, examination of tapes of early mother-infant interactions in pigmy chimpanzees and lowland gorillas, two of Homo sapiens' genetically closest primate relatives, offers some evidence for intuitive parent-like communication in the tactile mode, but an absence of the pattern of vocal and eye-to-eye visual exchange so characteristic of intuitive parenting in every human culture studied to date.

Mechanism and Genetics of Primate Vocalization

John Newman completed several studies investigating brain mechanisms involved in the production of specific vocal patterns in squirrel monkeys. The role of alpha-adrenergic receptors in production of isolation calls was examined by comparing the effects of different doses of the alpha antagonist clonidine, the alpha-2 antagonist yohimbine, and the alpha-1 antagonist prazosin. Call rate decreased in dose-dependent fashion with clonidine administration and increased with increasing doses of yohimbine. When a moderate dose of yohimbine was given concomitantly with the highest dose of clonidine, the suppressive effects of clonidine were reversed, but when prazosin, the alpha-1 receptor antagonist, was given concomitantly with clonidine, there was no reversal of call suppression, thus implicating alpha-2, but not alpha-1 receptors in isolation call production. The antidepressant imipramine also suppressed production of isolation call in these subjects. Another series of studies examined the effects of anticholinergic compounds on production of both isolation and alarm calls. Benactyzine increased alarm call production while decreasing the rate of isolation calling. These benactyzine effects could be blocked by pretreatment with the cholinemetic physostigmine (which crosses the blood-brain barrier) but not with the cholinmetic neostigmine (which does not cross the blood-brain barrier).

Newman also completed a long-term study of inheritance patterns of species-typical isolation calls in hybrid offspring of cross-species breeding between sympatric populations of two squirrel monkey species and between allopatric populations of the same species (Gothic-arch versus Roman-arch squirrel monkeys). Isolation calls of the hybrids resulting from sympatric crosses most closely resembled the characteristic Roman-arch phenotype, while calls of allopatric hybrids more closely resembled the Gothic-arch phenotype. A second long-term comparative study of isolation calls among members of the prosimian genus Lemur was also completed this year with the recording of calls from Lemur mongoz infants born at the Duke University Prosimian Primate Center. Comparative analyses of sound spectrographs revealed an increasing amount of broad-frequency noise over-riding the basic tonal structure of isolation calls from Lemur mongoz, Lemur fulvus, Lemur macaco, Lemur coronatus, and Lemur catta,

respectively. An additional study of vocal patterns in New World and prosimian primate species that routinely produce twin births was initiated with the recording of isolation calls from Cheirogaleus medius, Varrecia variegata, and Lemur fulvus infants (Lemuridae family), and from Cullithrix jacchus and Cebuella pygmae infants (Callitrichidae family).

Brain, Behavior, and Communication Studies

During the past year, the technical reach of these studies was greatly extended when David Symmes installed a new digital sound analysis system based on a DEC 11/73 computer equipped with color graphics. This system, which permits direct analysis of monkey calls at frequency ranges well beyond human hearing, has been effectively used to process audio tapes and to develop digital records suitable for sophisticated statistical analysis of the frequency domains of monkey vocalization as well as those observed in human mother-infant interactions, as described in the previous section. A recently acquired Kay digital sound spectrographic apparatus, with which the computer is interfaced, has given us a unique capability to analyze biological sounds.

Employing this new equipment, Symmes and Maxine Biben have collaborated on several studies of various elements of squirrel monkey communication, with the result that several important advances have been made in the study of squirrel monkey vocal signalling as a model language. Although the resemblance between any nonhuman primate vocal system and human language is distant, new developments in this field of study, and in this laboratory in particular, reduce the distance and provide insights about those linguistic properties which are highly conserved and general. For example, the quiet exchange of "chuck" calls between female squirrel monkeys with an established bond of friendship appears to be an excellent model of "conversations," i.e., vocal exchanges wherein the structure and information content of later utterances are modified by variables present in earlier vocalizing. Expanding on previous findings collected from Gothic-arch squirrel monkeys, these researchers have collected new data on chuck calls from Roman-arch subjects. This type of Saimiri is generally considered a subspecies, and among the best described phenotypic characteristics which distinguish them as a subspecies are vocal signals. Previous studies of subspecific differences were not extended to affiliative contexts, as in the present project. The new data suggest more similarities than differences between the subspecies' use of chucks, along with a few structural distinctions. One exception is the existence of a more rigid temporal "rule" regarding spacing of calls in Roman-arch animals. Affiliative partners respond very promptly or not at all to "question" chucks. It is possible that such temporal spacing contributes to recognition of familiar animals.

In the first of two new studies on the ontogeny of vocal communication in squirrel monkeys, it was found that "cackle" calls (used at other times and by adults) are given by the dominant partner in play bouts among subadult peers, and play "peep" calls (used only by young animals when playing) are given by the subordinate partner. When role reversal occurs, as it does frequently in play between similar age males, play peeps are given by both players. Thus usage corresponds with social relationship, as it does in the quiet affiliative context. The rate of play peeping and the structural complexity of the calls correlate with and in fact predict the duration of play bouts. The conclusion was drawn that play peeps reflect motivation to play rather than serving as

signals about the content of play or as metacommunicative reassurance to partners. The latter view, focusing in earlier literature on a hypothesized need to avoid a progression from play to fighting, is clearly inconsistent with these results, which are the first systematic study of playful vocalizations in a nonhuman primate.

A second ontogenic study nearing completion has addressed the question of early mother-infant vocal interactions, also in squirrel monkeys. It is widely believed that monkey vocalizations are preprogrammed and develop normally without a learning process. This belief has some support from descriptions of acoustic structure, but extension of the conclusion to functional use goes far beyond available evidence. The present initiative is intended to describe in some detail the nature of early vocal experience and usage and to follow functional development for several years. The study has already disclosed the preeminence in the first months of a call type not previously described, although probably heard by field observers. The "coax" call is used by mothers and juvenile females and results in the infant returning to the mother or aunt and, at times, to cause the infant to assume the nursing position. Pitch contours of this tonal call are being analyzed in terms of context, and they provide an unusual opportunity for comparison with similar data at the human level, as described in the previous section.

Child and Family Research

Frank Pedersen and his colleagues continued to work on two long-term projects this past year, and initiated three new studies investigating the development of nurturant responses by parents toward infants. The first long term project has involved the observational study of parent-infant interactions in a family context. One focus of the project has been on the effects of different types of short-term maternal separations on infant socio-emotional development. In families in which mothers began employment prior to their infants' third month of life, those infants judged to have secure versus insecure maternal attachment relationships at 15 months did not differ on any of four measures of the extent of employment-related separation they had experienced. However, a composite measure of continuity of substitute care was strongly related to secure/insecure attachment. Infants with a secure maternal attachment were equally distributed across the full range on the continuity scale. In contrast, 85% of infants with an insecure attachment experienced substitute care characterized by a lack of high continuity. These findings are the first to link factors in substitute care environments to the mother-infant attachment relationship. The question being addressed in ongoing research is whether lack of continuity in substitute care exerts a direct effect on the infant's attachment relationship, or whether this factor is a marker of a more basic characteristic of the mother, such as her sensitivity.

In studies of the father's role in the infancy period, follow-up observations were carried out of father-infant interactions at age one year wherein the fathers had evidenced contrasting affective reactions during the earlier months of parenthood. Men who reported in interviews that they had experienced periods of the "blues" or a dysphoric mood, as contrasted with men who did not report such feeling states, had been found in home observations at 3 months to have a more disengaged style of relating to their babies. They spent more time physically remote from the baby, touched their babies less frequently, and provided less frequent caregiving and affection than did the comparison group.

When their infants were 12 months old, these fathers displayed more engagement. In addition, a factor that was associated with the fathers' dysphoric mood when their infants were 3 months old, problems in the marital relationship, was reported by the men and their wives as significantly improved at age 12 months. These findings provide evidence of a compensatory process in men who had initial adaptational difficulties. The pattern of the findings is consistent with a transactional model of early experience that emphasizes the "self-righting" potential in human adaptations.

The second long term project, headed by Robert Klein, examined the nature and relative incidence of behavior problems in a sample of children receiving hormonal therapy for precocious puberty. Analyses completed this year focused on describing the combined behavioral influence of age of onset, duration of symptoms before onset of treatment, and pretreatment medical status as indicated by bone age, vaginal bleeding, relative height, Tanner stage, and, for the idiopathic precocious puberty group, the levels of gonadotropins and sex steroids. Age of onset and duration of symptoms appeared to be equally important variables, but the presence of vaginal bleeding showed no relationship to adjustment. Extensive correlational analyses were carried out to examine the combined effect of the various medical status indicators. In general, these factors had a synergistic effect, i.e., their influence was stronger when taking into account the other factors than when looking at their influence individually. Another finding is that estradiol levels are significantly related to adjustment, but none of estradiol's putative physiological effects (i.e., bone age, relative height, breast stage, and vaginal bleeding) significantly covaried with the adjustment measure. These studies are of great value in separating the effects of the elevated sex steroids on behavior and the effects of the sexual characteristics per se (i.e. precocious breast development), since the characteristics persist for some time after hormone antagonist therapy has been initiated.

Three new studies initiated this past year examine the development of parental behavior, especially that characterized as nurturant, toward infants. The first study addresses the intergenerational transmission of nurturant roles for females and males. The general hypothesis being tested is that beginning very early in their childrens' lives, parents communicate different expectations in their play behavior with male and female children regarding the care of babies, with mothers fostering stronger nurturant expectations than fathers, who in turn differentiate their role expectations for males and females more strongly than do mothers. The second study involves an intervention during the pregnancy for first-time expectant mothers. The intervention, which involves having the expectant mother (a) handle a young infant on three different occasions, (b) observing her behavior with the infant on videotape, and (c) receiving feedback about her behavior, was hypothesized to reduce anxiety, heighten the mother's sensitivity to different arousal states in the young infant, and facilitate her making appropriate responses to behavior in her own infant. The third study compares two groups of expectant parents, who did or did not experience the emotional sequalae of a previous pregnancy loss, in order to determine whether the loss (miscarriage, stillbirth, or neonatal death) contributes toward anxiety, depression, and a dysfunctional parental adaptation that could interfere with nurturant behavior toward the young. For each study, pilot investigations have been completed during the year and formal data collection is currently underway.

Laboratory of Comparative Ethology

Chief, *Stephen J. Suomi, Ph.D.

Senior Staff

John D. Newman, Ph.D.
*Frank A. Pedersen, Ph.D.
*David Symmes, Ph.D.

Fellows

Maxeen Biben, Ph.D.
Susan K. Theut, M.D.

Visiting Program

Yvonne Bryan, Ph.D.
Philip Goedeking, Ph.D.
Gunhild Kestermann, Ph.D.

Intergovernmental Personnel Act

Maria Michejda, M.D., D.M.Sc.

Guest Researchers

Phyllis W. Berman, Ph.D.
Sue Boinski, Ph.D.
Joseph Erwin, Ph.D.
J. Dee Higley, Ph.D.
Lynne C. Huffman, M.D.
Robert H. MacTurk, Ph.D.
Mary E. McCarthy, Ph.D.
Howard Moss, Ph.D.
Michelle Pato, M.D.
Kathlyn Rasmussen, Ph.D.
Vincent Smeriglio, Ph.D.
Anita M. Sostek, Ph.D.
Martha J. Zaslow, Ph.D.

Bibliography

Berman PW. Young children's responses to babies: do they foreshadow differences between maternal and paternal styles? In: Fogel A, Melson GF, eds. Origins of nurturance. Hillsdale, NJ: Lawrence Erlbaum Associates, 1986;25-51.

Biben M. Individual and sex related strategies of play wrestling in captive squirrel monkeys. Ethology 1986;71:229-41.

Higley JD, Suomi SJ. Parental behaviour in nonhuman primates. In: Sluckin W, Herbert M, eds. Parental behaviour. Oxford: Basil Blackwell, Ltd., 1986;152-207.

Hunter FT. Individual differences in adolescents' perception of interaction with friends and parents. J. Early Adolesc 1985;5:295-305.

Masataka N, Symmes D. Effect of separation distance on isolation call structure in squirrel monkeys (Saimiri sciureus). Am J Primatol 1986;10:271-8.

Messer DJ, McCarthy ME, McQuiston S, MacTurk RH, Vietze PM, Yarrow LJ. Relationships between mastery behavior in infancy and competence in early childhood. Devlop Psychol 1986;22:366-72.

Pedersen FA. Leon J. Yarrow (1921-1982). Am Psychol 1985;1137.

Pedersen FA, ed. Father infant relationship (translated into Japanese). New York: Praeger Publishers, 1986;218.

Rabinovich BA, Suwalsky JTD, Pedersen FA. The effects of maternal employment on infants: a pre test--post test design. J Genet Psychol 1986;147:283-5.

Shamma SA, Symmes D. Patterns of inhibition in auditory cortical cells in awake squirrel monkeys. Hearing Res 1985;19:1-13.

Sonis WA, Comite F, Pescovitz OH, Hench K, Rahn CW, Cutler G, Loriaux DL, Klein RP. Biobehavioral aspects of precocious puberty. J Am Acad Child Psychiatry 1986;25:674-9.

Suomi SJ. Anxiety like disorders in nonhuman primates. In: Gittlemann, ed. Anxiety disorders of childhood. New York: Guilford Press, 1986;1-23.

Suomi SJ. Behavioral aspects of successful reproduction in primates. In: Binerschke K, ed. Primates: The road to self sustaining populations. New York: Springer Verlag, 1986;331-40.

Suomi SJ. Response styles in monkeys: experiential effects. In: Siever LJ, Klar H, eds. Biological response styles: Clinical implications. Washington, DC: American Psychiatric Press, 1985;1-17.

Vietze P, McCarthy M, McQuiston S, MacTurk R, Yarrow L. Exploration in infants with Down syndrome. In: Tamir D, Russell A, Brazelton TB, eds. Intervention and stimulation in infant development. London: Freud Publication House, Ltd, 1986;62-70.

Laboratory of Developmental Neurobiology--
Phillip G. Nelson, M.D., Ph.D., Chief

This Laboratory investigates the neurobiologic mechanisms relevant to development of the nervous system, with emphasis on studies at the cellular, membrane and molecular levels. The basis for short- and long-term interaction between nerve cells is studied electrophysiologically and biochemically. Combined molecular and morphological methods are used in the analysis of experiential modifications of brain function and gene expression. Two primary interests have motivated much of the work done in the Section on Neurobiology led by P.G. Nelson over the past several years. The first reflects an attempt to understand the basic mechanisms responsible for excitatory synaptic transmission in the mammalian central nervous system. This group has been involved in studies on both synaptic excitatory transmitter release and the postsynaptic membrane response initiated by excitatory agents. A second area of interest concerns the roles that electrical and synaptic activity may play in shaping nervous system development. The mature functional architecture of synaptic circuitry in the mammalian brain is critically dependent on the pattern of activity imposed upon it during development by the structure of the environment. Nelson et al. have sought to understand some of the molecular and cellular processes involved in this coupling between environment stimuli and nervous system form and function. Obviously related in a general way, these two primary areas of interest have recently begun to reveal an important and experimentally relevant overlap of findings.

The Section on Neuroendocrinology, directed by D. Klein, has utilized the cells of the pineal gland as a system for analyzing in pharmacological, cell biological and molecular genetic terms the regulation of cell function produced by a neurotransmitter, norepinephrine. In receiving the NIH Director's award this year, Dr. Klein was cited "For pioneering studies of the pineal gland which have demonstrated the unique role of this organ in the neural regulation of metabolic and biological rhythms." He and his group have demonstrated the involvement of different adrenergic receptors and the complex interaction between these receptors as they are activated. They have developed techniques for directly addressing the cell biologic mechanisms of altered intracellular calcium ion concentration and second messenger activation that pineal cell stimulation induces. Klein et al. are currently attempting the molecular cloning of specialized pineal genes, which should allow the analysis of regulation at the level of transcription, and the availability of antibodies to several gene products has resulted in immunocytochemical and quantitative study of such proteins. Another major finding in the past year is that pineal cells send axonal projections into the central nervous system, so that a classically neuronal influence, as well as a neurohumoral effect, of the pineal on brain function may be anticipated.

The Unit on Molecular Neurobiology (B. Schrier) has continued its efforts to isolate and characterize specific genes involved in neuronal differentiation, using mouse neuroblastoma cell lines. Work is focused on the gene that encodes choline acetyltransferase (ChAT); sequencing of a gene tentatively identified as ChAT on the basis of colony selection (using an expression library with an anti-ChAT antibody) has not yet unambiguously established homology between the cloned sequence and the authentic gene. Other work is concerned with isolating the gene coding for a neurotrophic factor produced by brain tissue near a stab would in the rat cerebral cortex.

The work in Nelson's section may be divided into three projects, the first of which is focused on neuron-glial-neuron peptide mediated interaction. Powerful neuron-glial interactions with a major impact on nervous system development are proving amenable to detailed cell-biologic analysis in the mammalian cell culture systems employed in the Laboratory. D. Brenneman has shown that a neuropeptide, vasoactive intestinal peptide (VIP), is released from a subset of neurons in an activity dependent manner, and acts on non-neuronal cells, presumably glia, at concentrations as low as 10^{-12} M. A large increase in the release of several radioactively labelled, newly synthesized proteins can be demonstrated from glial cultures in response to VIP application. No such response occurs with neuronal cultures. This macromolecular material released by peptidergic stimulation of non-neuronal cells essentially abolishes the substantial neuronal death that normally occurs in culture in close approximation, both temporally and quantitatively, to the developmental loss of neurons that normally occurs in vivo. Exogenously supplied VIP produces the glial response and experiments with either anti-VIP antibodies or VIP fragments that block the VIP receptor convincingly demonstrate that endogenously released VIP has a similarly potent glia-mediated effect on neuronal survival. Closely related peptides with substantial homology to VIP either lack any such effects or are active only at substantially higher concentration (plausibly working through partial agonistic activity at the VIP receptor). These findings are particularly exciting in that they may provide a generalizable model for a major aspect of activity-dependent modification of nervous system development. (It is noteworthy that the widespread distribution of VIP-containing cells in the cerebral cortex would allow this sytem to subserve a broad developmental role therein). It seems unlikely, however, that VIP is the only agent that will prove capable of eliciting release of trophic material from glial cells, and some evidence exists that catecholamines may do so as well. These investigators hypothesize that a number of broadly distributed peptidergic and aminergic systems in the brain may have a specific developmental regulatory role mediated by the type of neuron-glial-neuron interactions that they have demonstrated. The culture systems allow detailed analysis of the neuron-derived signal acting on the glia receptors, the second messenger systems, and the neurotrophic materials that are released. The possible significance for understanding the basis of neurodevelopmental disorders is substantial.

A developmental effect of neural electrical activity, complementary to that described above, is its role in producing neuronal death. Many elegant studies have demonstrated in vivo that electrical activity is instrumental in the loss of neurons that is so characteristic of normal development. In this culture system, also, it can be shown that neuron survival in electrically active cultures treated with VIP is less than in electrically inactive cultures also treated with VIP or conditioned medium from VIP-treated glial cultures. (Electrically inactive cultures not treated with VIP or conditioned medium have the lowest neuronal survival).

A closely related area of study has to do with specific regulation of the development of central cholinergic neurons. The activity of choline acetyltransferase (ChAT) is increased in spinal cord cultures exposed to culture medium conditioned by glial cells. This group has shown, however, that activity related regulation of neuro-development is specific for different cell types. (Inhibitory neurons that take up gamma-amino-butyric acid are relatively insensitive to alterations in the state of electrical activity in the

cultures). It may be that different growth factors selectively affect different types of neurons. These workers have begun fractionation of glial-conditioned medium and will use the convenient ChAT activity assay system for initial evaluation of the different fractionation protocols. Comparison of ChAT stimulation activity with overall neuronal survival-promoting activity in selected fractions will be useful in identifying differential cell specific effects of different trophic molecules. Growth factors already identified, such as nerve growth factor, will be utilized in this regard as well. Identification of cholinergic neurons by an anti-ChAT antibody that has been characterized extensively will allow determination of the numbers of surviving cholinergic neurons as well as the aggregate enzymatic activity of the cultures under different experimental conditions. Experiments have begun on cholinergic development in cultures of the medial septal region of the brain, one of the two major sources of cholinergic efferents to the neo- and archicortex. The involvement of these systems in central information storage and in major neuropathologies makes direct experimental extrapolation of findings from the spinal cord to these systems of high priority.

E. Neale and P. Nelson have initiated a series of experiments to address the mechanism of electrical activity related to neuronal loss, and the closely related problems of synapse elimination and stabilization, using a compartmental culture system that should offer unique advantages for such an analysis. These experiments will interdigitate closely with the studies discussed above and, using the immunocytochemical and physiologic methods with which the lab has much experience, will be designed to test a number of specific hypotheses as to the cellular basis for the extensive synaptic remodelling and cell death that occur during development as a consequence of patterned neuronal electrical activity. Considerable methodological work will be involved in these studies, but this seems to be fully justified by the central role that these regressive processes play in the functional development of the nervous system.

A second project in the Section on Neurobiology concerns excitatory amino acids and postsynaptic receptor mechanisms. M. Mayer and G. Westbrook have now provided a relatively complete picture of the functioning of the major receptor systems mediating classical excitatory synaptic transmission in the central nervous system. A large body of heretofore paradoxical experimental results in the literature has been rationalized by the findings, widely cited in the field, of Mayer and Westbrook. At least two and probably three types of receptors have been demonstrated in experiments in vivo. These are termed, on the basis of agonist effectiveness, kainate, quisqualate or N-methyl-D-aspartate (NMDA) receptors.

NMDA receptors appear to be the most complex, and in a number of regards, the most interesting. The channels associated with this receptor are chemically gated, but exhibit a strongly voltage-dependent behavior which is due to blockade of the channels by magnesium ions. The functional morphology of the channels has been explored by measuring their conductance at various potentials in the presence of different divalent ions. The results suggest that an ion-binding site occurs in the channel with a selective affinity for the different ions. Binding of divalent cations reduces the permeability of the channels to monovalent cations, and the binding energy determines the voltage-dependent behavior of the channels. The NMDA channel has a relatively high permeability to calcium ions; the ratio of calcium to sodium permeability is approximately 5, an unusually high value for chemically gated channels. Measurement of

changes in intracellular calcium with calcium-sensitive dyes has demonstrated that NMDA receptor agonists produce an increase in intracellular calcium, while activation of kainate or quisqualate receptors do not show comparable changes. Other experiments confirm this unique calcium permeability feature of the NMDA receptor-associated channels.

This calcium ingress associated with NMDA receptor activation is of particular interest in view of the involvement of altered intracellular calcium in a variety of cellular regulatory processes. Thus, it is important to understand the relationship of these receptors to synaptic transmission in the central nervous system. Previous studies had implicated non-NMDA receptors in the mediation of fast excitatory synaptic transmission in most central synapses. Recent experiments in the LDN have demonstrated clearly, however, that most, if not all, spinal cord(SC) excitatory synapses involve a dual receptor mediated mechanism. Experiments utilizing manipulations of neuronal membrane potential, Mg^{++} ion concentration, and selective amino acid blockers, reveal that EPSPs are composed of a fast non-NMDA receptor mediated component and a much slower component, mediated by NMDA receptors. Since activation of NMDA receptors has also been implicated in several pathological situations (epilepsy and hypoglycemic brain damage), an understanding of their role in synaptic transmission is vital to an understanding of both normal and abnormal CNS function. Mayer and Westbrook have been successful in establishing tissue culture preparations wherein such synaptic mechanisms may be expected to be expressed as a longer term modification or plasticity of synaptic function. In particular, hippocampal neurons in vivo or in slice preparations exhibit long term potentiation of excitatory synaptic input under a variety of circumstances. Cultures of hippocampal neurons, in conjunction with their normal cholinergic input from the septal region, offer favorable opportunities for studying physiological mechanisms of neuronal plasticity in an extremely accessible experimental system.

Delta (δ) receptors could be demonstrated in all tested dorsal root ganglion (DRG) neurons and about two-thirds of SC neurons; activation of these receptors results in decreased transmitter output assayed electrophysiologically. Kappa (κ) receptors occur on about one-third of SC neurons, but only in co-occurrence with δ receptors. Mu (μ) receptors could be demonstrated only in a minor (<5%) proportion of the SC cells tested.

Tsien et al. have demonstrated three species of calcium channels of which the activation of one (the L channel) is increased by the dihydropyridine, BayK 8644. These investigators have now confirmed the agonist action of this agent in cultured DRG and SC neurons, and shown that BayK 8644 does not have a corresponding effect of increasing transmitter output from these cells. This suggests that L channels may not be involved in transmitter release from these cells. Mayer and Westbrook have found that nitrendipine, a blocker of voltage-sensitive calcium channels in many tissues, generally does not significantly affect calcium currents in cultured SC or DRG neurons. On average, no significant effects on transmitter output could be documented, although in a minority of cells some augmentation of output occurred with nitrendipine application.

In further work in this area, this group hopes to establish what specific type of calcium channel is involved in transmitter release. This would have impor-

tant neuropharmacological consequences, in that the different calcium channels are affected differently by different drugs.

The third project in the Section on Neurobiology concerns excitatory synaptic transmitter release mechanisms. Combined physiological and anatomical studies of cultured spinal cord neurons by E. Neale and P. Nelson have been directed at the question of the morphological identity of the physiological transmitter release element. While it appeared that the synaptic bouton corresponds to the release element, in some cases up to three-fourths of the boutons may be physiologically inactive, representing a functional "synaptic reserve" available for mobilization under appropriate circumstances. Inadequate regulation of intracellular calcium in some cell types or in some synaptic boutons may contribute to this functional inactivation; a raised intracellular calcium ion concentration would be expected to diminish the availability of voltage-sensitive calcium channels necessary for transmitter release. These investigators have now found that DRG neurons (as compared to SC neurons) are less able to maintain either transmitter output or voltage-sensitive calcium currents under conditions of sustained repetitive activation. These cells also appear to have a lower concentration of mitochondria in their synaptic boutons than do SC neurons.

Further pharmacological studies of calcium currents and transmitter release have utilized several opiate peptides and dihydropyridine agonists and antagonists of voltage-sensitive calcium channels. This group is especially interested in the selectivity of the distribution of presynaptic receptors on tissue-cultured neurons, and the question of the type of calcium channels that are responsible for transmitter release.

The findings of Mayer and Westbrook on excitatory synaptic mechanisms have clear relevance for the activity-dependent developmental modulations being analyzed by Brenneman's group. Pharmacological tools are available to address the question of which class of amino acid receptors may be involved in those developmental phenomena.

The Section on Neuroendocrinology, directed by D. Klein, has focused on cyclic nucleotide regulation in the pineal. Noradrenergic neural stimulation of the pineal gland is associated with dramatic regulation of melatonin synthesis by the gland. Both α-1 and β-1 adrenoreceptors are involved in this regulation, and considerable understanding has been achieved of the cell biological mechanisms activated via these two receptors and of the intricate interactions between them. The research program of the section has produced much new information of how α-1 and β-1 receptors interact to regulate cAMP and cGMP, which are directly involved in control of melatonin metabolism. Activation of the β adrenoreceptors is a prerequisite for stimulation of either cAMP or cGMP levels, but pure β-receptor stimulation has only a small effect on both cyclic nucleotides, representing less than 5% of the maximal response. α-1 receptor stimulation has no effect on either cyclic nucleotide by itself, but simultaneous stimulation of both receptors results in an elevation of cAMP and cGMP more than 100-fold greater than control values. It is of interest that the peptide, vasoactive intestinal peptide (VIP), that Brenneman is studying in neuronal and glial cultures, can mimic the effects of β-adrenergic stimulation of pineal cells.

This lab has made considerable progress in elucidating the role that calcium and phospholipids play in cAMP and cGMP regulation. Activation of the α-1 receptor produces a sustained increase in intracellular calcium due to an increase in net influx of calcium across the surface membrane through a ligand-activated, nifedipine-insensitive channel. The α-1 activation is accompanied also by an increase in calcium-dependent phospholipase C activity, which generates diacylglycerol from phospholipids. These changes in cytosolic calcium and diacylglycerol in turn result in activation of protein kinase C by translocating it from cytosol to the membrane of the pinealocyte. Protein kinase C probably acts via adenylate and guanylate cyclase and via GTP binding proteins, to increase levels of the cyclic nucleotides. An additional mechanism involving phospholipase A_2 and arachidonic acid has been implicated in regulation of cGMP. Mepacrin, an inhibitor of phospholipase A_2, blocks adrenergic stimulation of cGMP. α-1 receptor stimulation of phospholipase A_2 may be mediated by protein kinase C, since activation of this kinase activates the phospholipase. According to these findings, then, the cascade of events leading to an increase in cGMP may first involve α-1 stimulation of calcium ingress and diacylglycerol production (resulting from phospholipase C activation), followed by protein kinase C activation which stimulates phospholipase A_2. Increased activity of this enzyme results in production and release of arachidonic acid; this agent, or an active metabolite, appears to fully activate β-1 sensitive membrane-bound guanylate cyclase. This line of work will be actively pursued and is deemed important in that relatively little information is available regarding the molecular basis for cGMP regulation in neural tissue.

Continued adrenergic stimulation of the pineal gland results in desensitization, and recent findings in this lab indicate that this may involve protein kinase C; activators of this enzyme not only have stimulatory effects but also produce desensitization to adrenergic agonists. Long term effects seen in pinealocytes deprived of adrenergic stimulation are also of interest to the section. In this case, a large shift in responsivity of the gland to adrenergic stimulation is seen such that cAMP responses dominate over cGMP responses.

Cellular mechanisms related to regulation of the pinealocyte membrane potential are of significance in that norepinephrine (NE) produces a membrane hyperpolarization, and this hyperpolarization appears to be required for the adrenergic stimulation of N-acetyltransferase activity and of melatonin production. Ouabain or high K^+ block such stimulation. Studies of a ouabain-sensitive Na-K-ATPase have shown that this enzyme is absent in pinealocytes at birth and only develops postnatally. Correspondingly, ouabain does not block the adrenergic stimulation of N-acetyltransferase activity in pinealocytes at birth, although a high concentration of K^+ does. Interestingly, activation of phospholipase A_2, acting through the generation of fatty acids, appears to affect the transmembrane position of Na, K-ATPase, resulting in a 3-fold increase in the number of external, ouabain binding sites, with total inhibition of internal, catalytic sites. Thus adrenergic activation of phospholipase A_2 might decrease Na pump activity, depolarizing the pinealocyte.

A second project in the Section on Neuroendocrinology involves melatonin regulation. cAMP increases the production and release of melatonin by increasing the activity of one of the enzymes involved in the conversion of serotonin to melatonin. This is a two-enzyme pathway involving an N-acetyltransferase and an

O-methyltransferase. N-acetyltransferase regulates the daily rhythm in melatonin synthesis, and is regulated by norepinephrine acting through a cAMP mechanism. cAMP stimulates the activity of rat N-acetyltransferase 100-fold. This stimulation involves new synthesis of protein and gene expression, and is rapid, with a doubling time of about 15 minutes. cAMP also stabilizes N-acetyltransferase in an active form, preventing inactivation. When cAMP decreases, N-acetyltransferase activity decreases rapidly, with a halving time of about three minutes. Hydroxyindole-O-methyltransferase is also regulated by norepinephrine. However, it changes gradually in response to stimulation, and changes are detected only following a week of treatment.

As indicated above, the adrenergic-cyclic AMP stimulation of N-acetyltransferase requires membrane hyperpolarization. Some recent studies have indicated that depolarization by K^+ blocks gene expression, and thereby blocks the increase in N-acetyltransferase. Ouabain appears to block the increase in N-acetyltransferase at a latter step, allowing gene expression but appearing to prevent translation. This line of investigation is of special importance because it is a rare example of a role of membrane potential in gene expression in the nervous system, and will be important to pursue.

The detailed study of the regulation of N-acetyltransferase and hydroxyindole-O-methyltransferase activity in the pineal gland is important because it will provide some insight into the adrenergic regulation of gene expression in the nervous system. Up to this time, studies have been almost entirely limited to the analysis of enzyme activity, with no meaningful measurement of enzyme protein or mRNA, or related studies on post-transcriptional and post-translational processing. These studies have not been possible because the appropriate probes have not been available. This lab has now begun a major effort to obtain the tools needed to study the regulation of N-acetyltransferase and hydroxyindole-O-methyltransferase, and related proteins of interest. Highly purified preparations of sheep N-acetyltransferase have been obtained in sufficient amounts to sequence the enzyme and initial steps have been taken to raise antiserum. The bovine enzyme, hydroxindole-O-methyltransferase, has been prepared in suitable amounts of highly pure material so that the sequence of this protein is now being determined; a partial sequence has been established. High titre polyclonal antiserum with high specificity has been prepared in rabbits, and an extensive characterization has indicated that this is a reliable means of detecting the bovine enzyme.

To prepare cDNA probes for N-acetyltransferase and hydroxyindole-O-methyltransferase, a bovine pineal cDNA library was prepared in collaboration with the Unit on Molecular Neurobiology in the LDN. An improved bovine pineal cDNA library is now being prepared, along with a day-sheep and night-sheep pineal cDNA library, and a control-rat and adrenergically-stimulated rat pineal cDNA library. These will be used to clone specific cDNA's of interest, for eventual experimental use in the analysis of gene expression in the pineal gland. In an associated effort to study the regulation of gene expression in the pineal gland, work has been done on the isolation of the S-antigen gene. The S-antigen, a putative cyclic nucleotide regulatory protein, is found only in the pineal gland and retina.

A number of observations indicate that the paraventricular nucleus of the hypothalamus may be involved in the circuits which control the release of norepinephrine from the sympathetic nerve endings in the pineal gland. This

past year, the Section has collaborated with workers at the University of Pennsylvania, Department of Psychology, to show that electrical stimulation of the paraventricular nucleus during the day causes an increase in melatonin production. This work is continuing with iontophoresis of the putative transmitters.

In collaboration with H. Korf, Geissen, West Germany, the Section reported this year that there are neural connections between the pineal gland and both the habenula and posterior commisure. This is a very significant discovery because it offers a reason to suspect that the pineal gland might communicate directly with other areas of the brain, not only by humoral avenues. The work on pineal projections has continued in a collaborative effort with Drs. Ungerleiter and Mishkin, NIMH, using the monkey. Preliminary findings point to the possibility that such connections are also present in primates. This work may be critical in future research on the pineal gland.

The Molecular Neurobiology Unit, led by B. Schrier, has contined its work with differentiation-stage-specific cDNAs from mouse neuroblastoma cells, and from a hybrid cell line (mouse neuroblastoma x rat glioma). To date, several differentiation-specific and differentiation-enriched clones have been isolated, and the degree of specificity confirmed by Northern blots and/or dot blots. RNA blots are also being used to determine the environmental stimulus responsible for activation of the expression of these genes, and the timing of that activation during the differentiation paradigm.

These investigators have recently initiated an effort to determine whether the cDNA clone designated λChAT7 is homologous to the mRNA which encodes the enzyme choline acetyltransferase. By means of *in vitro* translation of hybrid-selected mRNA, followed by assays for enzyme activity and Western blot identification with monoclonal and polyclonal antibodies, they are trying to obtain additional evidence for this clone's identity.

This group has also employed the cascade hybridization technique to partially purify cDNAs enriched in rat cerebral cortical tissue near a stab wound 10 days afte the lesion. A library of these cDNAs cloned in an expression vector has been obtained, is being characterized as to its specificity for lesioned brain, and will be screened for a neurotrophic factor necessary for the survival of sympathetic neurons in culture. This factor is known to be much enhanced in lesioned brain tissue.

Schrier et al. have achieved the expression of mouse pro-opiomelanocortin (POMC) in *E. coli*. The 5'-untranslated region of a full-length POMC cDNA clone was exonucleolytically deleted, and the coding sequence was inserted into a bacterial expression vector within which transcription of the POMC coding sequence is under the control of the *lac* operator. Cells that harbor the clones can be induced to synthesize large amounts of the POMC protein. POMC analogues will be produced from specifically mutagenized clones and, in collaboration with the Cellular Neurobiology Unit (LNN), an investigation will be undertaken on the basis of the cleavage-site specificity of a pro-hormone converting enzyme (using analyses of the cleavage products produced by reaction of the converting enzyme with the POMC analogues).

Primary cultures of glial cells have been observed by others in the Laboratory of Developmental Neurobiology to secrete a neuronotrophic factor that stimulates choline acetyltransferase activity in fetal mouse spinal cord neurons.

Schrier and his colleagues have observed that Xenopus laevis oocytes that have been injected with glial cell-derived RNA will secrete a neuronotrophic factor that exhibits the same properties as that detected in glial cell conditioned medium (mock-injected oocytes do not produce the factor). Hence these workers now have an assay for the messenger RNA that encodes the factor, and have undertaken the molecular cloning of its cDNA.

Laboratory of Developmental Neurobiology

Chief, *Phillip G. Nelson, M.D., Ph.D.

Senior Staff

*David Klein, Ph.D.
Elaine Neale, Ph.D.
Bruce Schrier, M.D., Ph.D.

Fellows

Ralph F. Alderson, Ph.D.
Douglas E. Brenneman, Ph.D.
Douglas Kniss, Ph.D.
Gary L. Westbrook, M.D.

Visiting Program

Yu Chang, M.D.
Ian D. Forsythe, Ph.D.
Anthony K.-C. Ho, Ph.D.
Mark L. Mayer, Ph.D.
Kazuhide Sugiyama, M.D.

Experts

Eugene T. Butler, Ph.D.

Guest Researchers

Mildred Brammer, Ph.D.
Valentin Cena, M.D., Ph.D.
Carmen Gonzalez, M.D.

Bibliography

Brenneman DE. Role of electrical activity and trophic factors during cholinergic development of dissociated culture. Can J Physiol Pharmacol 1986;64:356-62.

Brenneman DE, Eiden LE. Vasoactive intestinal peptide and electrical activity influence neuronal survival. Proc Natl Acad Sci 1986;83:1159-62.

Brenneman GE, Eiden LE, Siegel RE. Neurotrophic action of VIP on spinal cord cultures. Peptides 1985;6:35-9.

Clements JD, Nelson PG, Redman SJ. Intracellular tetraethylammonium ions enhance group Ia excitatory postsynaptic potentials evolved in cat motoneurones. J Physiol (Lond) 1986;377:267-82.

Habig WH, Bigalke H, Bergey GK, Neale EA, Hardegree MC, Nelson PG. Tetanus toxin in dissociated spinal cord cultures: long term characterization of form and action. J Neurochem 1986;47:930-7.

Handelmann GE. Neuropeptide effects on brain development. J. Physiol, Paris 1985;80:268-74.

Handelmann GE. Regulation of brain opiate receptors during development: consequences for adult behavior In: Biggio G, Spano PF, Toffano G, Gessa GL, eds. Modulation of central and peripheral transmitter function. Fidia Research Series, Symposia in Neuroscience III. Padova, Italy: Liviana Press, 1986:469-71.

Handelmann GE, Dow-Edwards D. Modulation of brain development by morphine: effects on central motor systems and behavior. Peptides 1985;6:29-34.

Jia M, Nelson PG. Calcium currents and transmitter output in cultured spinal cord and dorsal root ganglion neurons. J Neurophysiol 1986;56:1257-67.

Ho AK, Somers RL, Klein DC. Development and regulation of rhodopsin kinase in rat pineal and retina. J Neurochem 1986;46:1176-9.

Klein DC. Photoneural regulation of the mammalian pineal gland. In: Short R, eds. Photoperiodism, melatonin and the pineal. Ciba Foundation Symposium. London: Pittman, 1986;117:38-56.

Klein DC, Sugden D, Vanecek J, Thomas TP, Anderson WB. Phospholipid-protein kinase C involvement in the adrenergic regulation of pinealocyte cycle AMP: a model of how modulators act. In: Horrocks LA, Freysz L, Toffano G, eds. Phospholipids research and the nervous system. Biochemical and molecular pharmacology. Padova: Liviana Press, 1985;113-20.

Klein DC, Voisin P, Namboodiri MAA. The pineal family of aromatic amine N-acetyltransferase. BioEssays 1985;3:217-9.

Korf H-W, Klein DC, Zigler, JS, Gery I, Schachenmayr W. S-antigen-like immunoreactivity in a human pineocytoma. Acta Neuropathol 1986;69:165-7.

Korf H-W, Oksche A, Ekstrom P, Gery I, Zigler, Jr JS, Klein DC. Pinealocyte projections into the mammalian brain revealed with S-antigen antiserum. Science 1986;231:735-7.

Litzinger MJ, Foster G, Brenneman DE. [H^3]-Nitrendipine binding in non-neuronal cell cultures. Biochem Biophys Res Commun 1986;136:783-8.

Litzinger MJ, Lombet A, Brenneman DE, Lazdunski M. [^3H]-Tetrodotoxin binding in neuronal and non-neuronal spinal cord cultures. Biochem Biophys Res Commun 1986;138:1250-6.

Litzinger MJ, Nelson PG, Pun RYK. Effects of nitrendipine on the voltage-sensitive calcium channel in mammalian sensory neurons. J Neurosci Res 1985;14:415-22.

Mayer ML. Selective block of inward but not outward rectification in rat sensory neurones infected with herpes simplex virus. J Physiol 1986;375:327-38.

Mayer ML, James MH, Russell RJ, Kelly JS, Pasternak CA: Changes in excitability induced by herpes simplex viruses in rat dorsal root ganglion neurons. J Neurosci 1986;6:391-402.

Mayer ML, Westbrook GL. Excitatory amino acids: membrane physiology. In: Rogawski, MA, Barker JL, eds. Neurotransmitter actions in the vertebrate nervous system. New York: Plenum Press, 1985;125-39.

Nelson PG, Pun RYK, Westbrook GL. Synaptic excitation in culture of mouse spinal cord neurones: receptor pharmacology and behaviour of synaptic currents. J Physiol 1986;372:169-90.

Pun RYK, Neale EA, Buthrie PB, Nelson PG. Active and inactive central synapses in cell culture. J Neurophysiol 1986;56:1242-56.

Sher PK, Neale EA, Machen VL. Autoradiographic localization of benzodiazepine receptor binding in dissociated cultures of fetal mouse cerebral cortex. J. Neurochem 1986;46:899-904.

Sugden AL, Sugden D, Klein DC. Essential role of calcium influx in the adrenergic regulation of cAMP and cGMP in rat pinealocytes. J Biol Chem 1986;261:11608-12.

van Veen, T, Elofsson R, Hartwig H-G, Gery I, Mochizuki M, Cena V, Klein DC. Retinal S-antigen: immunocytochemical and immunochemical studies on distribution in animal photoreceptors and pineal organs. Exp Biol 1986;45:15-25.

van Veen T, Ostholm T, Gierschik P, Spiegel A, Somers, R, Korf HW, Klein DC. Alpha-transducin immunoreactivity in retinae and sensory pineal organs of adult vertebrates. Proc Natl Acad Sci 1986;83:912-6.

Vanecek J, Sugden D, Weller JL, Klein DC. See-Saw signal processing in pinealocytes involves reciprocal changes in the $alpha_1$-adrenergic component of the cyclic GMP responsse and the beta-adrenergic component of the cyclic AMP response. J Neurochem 1986;47:678-86.

Laboratory of Theoretical and Physical Biology--
David Rodbard, M.D., Chief

This Laboratory conducts a wide range of computational and theoretical studies, applying mathematical, statistical, and computer-based techniques to the analysis of complex clinical, biological, and pharmacological problems. Experimental work in the Laboratory involves the study of receptor-ligand interactions; the mass spectroscopic analysis of metals, steroids, and amino acids in man; and the physical-chemical characterization of peptides using polyacrylamide gel electrophoresis and related approaches.

The section directed by D. Rodbard analyzes the interaction of hormones and neurotransmitters with their receptors using a combined theoretical and experimental approach. With this approach, the group has previously demonstrated the existence of the mu-1 subtype of opioid receptor, which is responsible for analgesia and the hypothalamic control of pituitary function. These investigators have also characterized other multiple subtypes of the opioid receptors in adrenal medulla, designated K1, K2, and K3. These findings have been made possible by the development of new mathematical and statistical methods for studying dose-response curves in the presence of multiple ligands (the "multi-ligand" design); the combination of modelling and biochemical approaches; and computerized methods for optimization of experimental design, analysis and validation of results.

Using similar methods, Rodbard and his colleagues have now demonstrated and characterized at least three types of oxytocin and vasopressin receptors throughout the male genital tract of the pig. Oxytocin receptors are most concentrated in the testis of the prepubertal pig, and this concentration decreases progressively in the epididymis and vas deferens, whereas the reverse pattern of localization is observed for the V_1 vasopressin receptor. A pharmacologically distinct type of vasopresin receptor, designated V3, is present at extremely high concentrations in the seminal vesicle, is coupled to adenylate cyclase, and resembles the vasopressin receptor of the anterior pituitary. The presence of these receptors implies a physiological role for oxytocin and vasopressin in male reproduction.

As noted above, this group had earlier demonstrated the presence of three distinct subtypes of kappa receptors in bovine adrenal medulla, and they are currently studying the effects of opiates on the regulation of catecholamine release. They have demonstrated the presence of both high and low affinity opioid receptors in neurosecretosomes from bovine posterior pituitary (in collaboration with the Laboratory of Neurochemistry and Neuroimmunology), and have shown that these are exclusively of the kappa type. The demonstration of these multiple opioid receptor subtypes now mandates a thorough reevaluation of the pharmacology, biochemistry and function of opioid receptors. These studies will provide a basis for the development of new selective agonists and antagonists which in turn will be necessary for further dissection of these intricate systems which appear to control neurotransmitter and endocrine systems. Finally, these investigators also studied the influence of saturated and unsaturated fatty acids on the binding of opioids, ouabain, and beta adrenergic ligands to their receptors, to evaluate the specificity of the physiological effects reported in such experiments.

During the past year, this section also developed a new, universally applicable method for curve-fitting which combines the advantages of empirical methods (simplicity, versatility, flexibility, no need to provide a model or equation) with several of the advantages of mathematical modelling (objectivity, statistical hypothesis testing, standard errors of critical parameters, computerization and automation). This method promises to have widespread utility in scientific data analysis.

Objective statistical methods for detection and characterization of episodic pulsatile hormone release have also been developed, and applied to a wide range of endocrine systems. When instantaneous hormone secretion is computed, it appears that > 90% of the "tonic" secretion of luteinizing hormone (LH) in man occurs in bursts lasting less than 10% of the time. This result will have considerable impact on our understanding of the physiology, not only of LH, but of many other peptide and steroid hormonal systems.

A major recent accomplishment has been the development of several innovative computer programs to assist physicians, paramedical personnel and diabetic patients with home monitoring of blood glucose and self-adjustment of insulin dosage. These programs, for the IBM-PC and Apple II classes of microcomputers, provide data storage and retrieval, graphical and statistical analyses, and "expert" consultation regarding adjustment of insulin dosage, and timing of glucose measurements. The programs also alert the patient to the need for conversation with a physician. Human engineering factors have been optimized to facilitate acceptance, and the programs have multiple fail-safe features. Rodbard's group has worked out a general flexible approach for the development of customized treatment plans ("algorithms") for individual patients. The computer permits examination of the discrete and continuous "glucose profile" to evaluate median circadian patterns, or changes by day of week with appropriate weighting and smoothing. The program provides a detailed analysis and interpretation of the glucose profile. The ancillary documentation for the programs has developed into a book, explicitly describing a versatile new approach to management of patients with type-I diabetes. These programs are now being evaluated in controlled clinical trials at medical centers throughout the country, with special reference to their use in children, adolescents, and during pregnancy.

P. Munson devised a novel statistical analysis of an SV40-based DNA damage/repair system (a shuttle vector) in order to compare several possible models of the mammalian DNA repair-mutagenesis process (in collaboration with the Section on Viruses and Cellular Biology). Results showed that insertion of one base (adenine) occurs with higher probability opposite a damaged base in the template DNA strand, regardless of the identity of the original base at that location.

Munson also developed a new statistical test and computer program for evaluating the "goodness-of-fit" of a mathematical model to observed data, based on a mathematical characterization of random noise as "rough" and the signal as "smooth."

The section directed by A. Chrambach has made progress in the development of a method for steady-state isoelectric focusing and two-dimensional macromolecular mapping. These workers have found that combined use of Immobiline pH gradients

and soluble carrier ampholytes provides pH-gradient stability, with improved entry of proteins into the gel and uniformity both of conductance and the voltage gradient. The procedure has now been streamlined so that it is entirely practical.

Agarose gels and highly crosslinked polyacrylamide gels have been applied to the fractionation and size-charge characterization of viruses. The "Ferguson plots" of log(mobility) versus gel concentration are nonlinear and complex: Mathematical modelling has been used to derive the apparent properties of the gel, and to permit reliable estimation of particle size (radius) and free mobility. In addition, slope changes in the Ferguson plot were used as a measure of the malleability of the particle. Analysis of the physical properties of viruses and cellular organelles was based, for the first time, on commercially available standards (polystyrene microspheres with diameters determined by electron microscopy), by which the pore size and other properties of the gels were calibrated.

Several of the practical applications of Chrambach's approach to macromolecular analysis achieved during the past year follow: Using agarose gel-electrophoresis, this group determined the particle size of covalently linked protein-polysaccharide conjugates (e.g., meningococcus) to be used as experimental vaccines (see description of work in the LDMI). These physical measurements will be important if they correlate with the degree of immunogenicity of these preparations, to be employed in the prevention of invasive bacterial infections in children. In another study, Chrambach and his colleagues demonstrated the applicability of quantitative agarose gel-electrophoresis to the analysis of sub-cellular particles--here, clathrin-coated vesicles from brain and liver. The method appears promising for analytical and preparative studies on vesicles involved in metabolite traffic across cell membranes in general. During the past year also, Chrambach and his colleagues employed quantitative polyacrylamide gel-electrophoresis and isoelectric focussing to demonstrate that in hypertensive disease, wherein inactive renin accumulates in the circulation, the block responsible is at the level of the enzymatic cleavage of prorenin to renin. It may be possible to target this block for pharmacologic intervention. Further, Chrambach's group, using PAGE with glutaraldehyde crosslinking of the peptides prior to staining, was able to separate angiotensin I, II, and III.

The separability of these three peptides demonstrates applicability of the method to peptide separation and isolation in general. Chrambach's work on the theoretical analysis of isoelectric focusing, using multiple buffer constituents, has led to a substantial advance for all studies which rely on isoelectric focusing, and in particular, the widely applied practice of two-dimensional macromolecular mapping which aims at the detection of minor component changes (characteristic of developmental changes) against the background of a constant multi-component pattern. Finally, Chrambach and his colleagues have been pursuing the development of a rapid, continuous electrophoresis apparatus that would allow physical characterization of each species in a complex mixture, thus permitting the multi-component analysis of biological fluids in clinical samples, as well as industrial quality control. During the past year, Chrambach's group found that non-crosslinked liquid polyacrylamide possesses a sieving capacity similar to that of crosslinked gels; with this finding, the capillary apparatus becomes applicable to molecular and particle sieving, with optical analysis and on-line computer evaluation of migration rates. Redesign of the capillary apparatus has permitted very rapid size and net charge estimates for each component.

The group led by A. Yergey has made several important advances in the application of liquid-chromatography-thermospray mass spectrometry (LC-MS) to compounds of biochemical and clinical interest. New methods have been developed for measurement of deuterated glucose and cortisol in human plasma, and are now being applied to clinical investigation of the role of cortisol metabolism in normal puberty, as well as to biochemical analysis of the pathophysiology of various inborn errors of metabolism (e.g., glycogen storage diseases). Studies of metabolism of stable isotopes of calcium in neonates (to examine the role of vitamin D supplements), in pregnancy, lactation and in disorders with ectopic calcification are continuing with collaborators at NIH and throughout the country. LC-MS has now been applied to a host of compounds, including the carnitine derivatives important in fatty acid and drug metabolism, studies of glucuronide derivatives of drugs, and a novel 1-amino acid (hypusine). Pilot studies have indicated the feasibility of new approaches to characterization of oligo- and small polypeptides, and these methods are now being applied to growth factors derived from neoplasms.

As noted above, one principle objective of Yergey's work is to employ mass spectrometry to elucidate the kinetics of calcium metabolism in normal children and adults as well as to evaluate disease-related changes in this metabolism. Stable isotopes make such studies possible in children and in women of childbearing age, studies that are not possible with radioactive calcium tracers; stable tracers also permit repeated measurements. Yergey and his colleagues employ thermal ionization-isotope ratio-mass spectrometry together with a quadrupole mass filter in order to measure tracer enrichments in serum, urine, etc. The isotope ratio measurements are analyzed with a multi-compartmental mathematical model. Two stable isotopic tracers, one given i.v. and the other orally, are used which permit studies on absorption as well as excretion. During the past year, Yergey has refined the instrumentation such that he can now detect a 2% change in the natural ratio of $^{43}Ca/^{48}Ca$. Population statistics have been obtained in a group of normal children for the mean residence time of calcium in plasma (11.1 $\pm$ 2.5 hrs) and the fraction of calcium absorbed from the diet. These studies have now been extended to pregnancy and lactation.

Another objective of Yergey's group is to develop and apply new methods for analysis of biological materials that require mass spectrometric analysis, but which have not previously been amenable to such analysis by reason of volatility, thermal lability, or charge state. The basic approach involves a direct interface of high performance liquid chromatography effluent with the mass spectrometer source. Ions are desorbed directly from vapor droplets that are heated rapidly during passage from the HPLC capillary through the ion source by a mechanism that resembles other desorption techniques. Thermospray LC/MS has the important advantage over other methods because of a) a chromatographic inlet, b) applicability to analysis of mixtures, and c) simplicity of sample preparation. Recent applications include the identification of novel fatty acid conjugates of carnitine in patients with Reye's syndrome, organic acidurias and valproic acid toxicity; the separation and quantitation of cortisol in human plasma; and the separation and quantitation of glucose as an aspect of turnover studies in glycogen storage disease.

Within the past year, methods have been further improved for the analysis of cortisol, its metabolites and biosynthetic precursors, as well as testosterone, vitamin D, glucose, fructose, sorbitol, and acetylcholine. These methods have been applied clinically to the further study of glycogen storage diseases,

Cushings' syndrome, and hormonal changes during puberty. A new metabolite of cortisol, 11-hydroxyandrostenediol, has been discovered. This steroid is produced by an enzyme found in erythrocytes, and may be responsible for a variant of Cushings' syndrome that occurs in the presence of normal levels of cortisol. A new method for measurement of gastrointestinal calcium adsorption in the human neonate has also been developed, and is being applied to the evaluation of the optimal levels of vitamin D in the nutrition of premature infants.

In summary, the LTPB has had an extremely productive year, with its wide ranging multidisciplinary program for the development of new strategies, biochemical methods, and computer programs to permit improved analysis of ligand-receptor systems; for purification and characterization of protein and polypeptide hormones; for studies on the metabolism of metals and organic compounds in man and in vitro; and for more effective utilization and interpretation of laboratory and clinical data.

Laboratory of Theoretical and Physical Biology

Chief, *David Rodbard, M.D.

Senior Staff

*Andreas Chrambach, Ph.D.
Peter Munson, Ph.D.
*Alfred Yergey, Ph.D.

Visiting Program

Nora V. Esteban, M.D.
John S. Fawcett, Ph.D.
Laszlo Orban, Ph.D.
Enrico Rovati, M.D.
Siegfried Schwarz, M.D.
Dietmar Tietz, Ph.D.

Guest Researchers

Birgit An der Lan, Ph.D.
Vincenzo Guardabasso, M.D.
James E.A. McIntosh, Ph.D.
Samuel Sadikario, M.D.
Elisabeth Hahn, Ph.D.

Bibliography

An der Lan B, Cowell JL, Burstyn DG, Manclar CR, Chrambach A. Characterization of the filamentous hemagglutinin from bordetella pertussis by gel electrophoresis. Mol Cell Biochem 1986;70:31-55.

Chrambach A, Bocek P. Directions of an electrophoretic methodology responsive to the analytical needs of biochemistry. Trends in Analyt Chem 1985;4:224-9.

DeFeo ML, Maggi M, Guardabasso V, Rodbard D, Delitala, G, Fazzi V, Genazzani A, Facchinetti F, Forti G. Naloxone administration does not affect gonadotropin secretion in agonadal men either basally or during testosterone treatment. J Clin Endocrinol Metab 1986;63:257-61.

Fawcett JS, Chrambach A. Development of stationary 2-dimensional gel patterns. In: Dunn MJ, ed. Electrophoresis '86. Weinheim: VCH Publishers, 1986;569-73.

Fawcett JS, Chrambach A. Diagonal isoelectric focusing on immobilized pH gradients: concept and preliminary performance tests. Electrophoresis 1986;7:153-8.

Fawcett JS, Chrambach A. Polyacrylamide gel electrofocusing in pH gradients formed by carrier ampholytes and by immobiline: pro's and con's. In Reeder DS, ed. Proceedings of the 1986 Meeting of the Americas Branch of the Electrophoresis Society. Gaithersburg: National Bureau of Standards, 1986; NBSIR 86-3345;13-35.

Fawcett JS, Chrambach A. Simplified procedure for the preparation of IPG-gels. Electrophoresis 1986;7:260-5.

Fawcett JS, Chrambach A. The voltage across wide pH range IPG gels and its modulation through the addition of carrier ampholytes. Electrophoresis 1986;7:266-72.

Guardabasso V, Munson PJ. Superbasic system expanded. Interface 1985;126:19-22.

Jaffe ML, Esteban N, Rodbard D. A tutorial for the SUGAR-][diabetes data management program. Bethesda: Division of Computer Research 1986;37.

Keane PM, Walker WHC, Thornton G, Rodbard D. Studies of thyroxine binding to plasma proteins in health and disease. Clin Biochem 1986;19:52-7.

Krumins SA, Lutz RA, Costa T, Rodbard D. Interaction of dimeric and monomeric enkephalins with NG108-15 hybrid cells: a kinetic analysis. Neurochem Res 1986;6:839-50.

Levinson PD, Goldstein MD, Munson PJ, Gill JR, Keiser HR. Endocrine, renal and hemodynamic responses to graded dopamine infusions in normal subjects. J Clin Endocrinol Metab 1985;60:821-6.

Liberato DJ, Millington DS, Yergey AL. Analysis of acylcarnitines in human metabolic disease by thermospray liquid chromatography/mass spectrometry. In: Castignoli N, Burlingame AL, eds. Elsevier-Holland, International Symposium on Mass Spectrometry in the Health and Life Sciences. San Francisco: Elsevier Science Publishers, 1985:333-48.

Liberato DJ, Yergey AL, Weintraub ST. Separation and quantification of choline and acetylcholine by thermospray liquid chromatography/mass spectrometry. Biomed Mass Spectrom 1986;13:171-4.

Liberato DJ, Yergey AL. Solvent selection for thermospray liquid chromatography/mass spectrometry. Anal Chem 1985;58:6-9.

Nocerini MR, Yost GS, Carlson JR, Liberato DJ, Breeze RG. Structure of the glutathione adduct of activated 3-methylindole indicates an imine methide as the electrophillic intermediate. Drug Metab Dispos 1985;13:690-4.

Oerter KE, Guardabasso V, Rodbard D. Detection and characterization of peaks and estimation of instantaneous secretory rate for episodic pulsatile hormone secretion. Comput Biomed Res 1986;19:170-91.

Pernick N, Jaffe ML, Esteban NV, Sung S, Rodbard D. Microcomputer programs to assist with home monitoring of blood glucose and self-adjustment of insulin dosage using flexible, customized algorithms. Diabetes 1985;34(suppl 1):138.

Pernick N, Rodbard D. Personal computer programs to assist with home monitoring of blood glucose and self adjustment of insulin dosage. Diabetes Care 1986;9:61-9.

Rachmel A, Hazelton GA, Yergey AL, Liberato DJ. Furosemide 1-O-Acyl Glucuronide. In vitro biosynthesis and pH-dependent isomerization to beta-glucuronidase-resistant forms. Drug Metab Dispos 1985;13:705-10.

Rodbard D. Interpolation of the RIA dose response curve according to models based on the law of mass action. Ligand Quarterly 1986;5:86-8.

Rodbard D. Radioimmunoassay curve-fitting mass action law models: A commentary [Letter to the Editor]. Ligand Quarterly 1986;5:86-8.

Rodbard D, Foy J, Munson PJ, Zweig M, Lewis T. A personal-computer-based system for acquisition and analysis of radioimmunoassay data. Proceedings, Symposium on Computer Applications in Medical Care. Silver Spring: IEEE Computer Society Press 1985;455-9.

Rodbard D, Lutz RA, Cruciani RA, Guardabasso V, Pesce GO, Munson PJ. Computer analysis of radioligand data: advantages, problems and pitfalls. In: Rapaka RS, Hawks RL, eds. Opioid peptides--molecular pharmacology, biosynthesis, and analysis. Washington DC: U.S. Government Printing Office, NIDA Research Monograph 1986;70:209-22.

Rodbard D, Munson PJ, Cole BR. Linear regression in medical research--The "BRIGHT-STAT-PACK" approach. [Letter to the Editor.] N Engl J Med 1986;315:197-8.

Tietz D, Chrambach A. Analysis of convex ferguson plots in agarose gel electrophoresis by empirical computer modeling. Electrophoresis 1986;7:241-50.

Tietz D, Chrambach A. Konsequenzen der Variablitaet der Zwei-faser-struktur der Agarose fuer die Interpretation and Praxis der Agarose-Elektrophorese. In: Radola BJ, eds. Elektrophorese Forum. Munich: Technical University of Munich 1985;165-71.

Tietz D, Gombocz E, Chrambach A. Particle dynamics revealed by agarose gel electrophoresis. In: Dunn MJ, ed. Electrophoresis '86. Weinheim: VCH Publishers, 1986; 253-8.

Yergey AL, Vieira NE, Covell DG, Muenzer J. Studies of human calcium kinetics with stable isotopic tracers. In: Muccino RR, ed. Synthesis and applications of isotopically labelled compounds-1985. Amsterdam: Elsevier Science Publishers, 1985;343-8.

Human Genetics Branch--
Michael A. Zasloff, M.D., Ph.D., Chief

This Branch carries out research to elucidate the pathophysiology of human genetic and developmental disorders through an understanding of basic biological mechanisms. Clinical research projects include studies on the natural history, treatment, and methods of diagnosis of several heritable disorders of man.

During the past year, work in the section directed by M. Zasloff has concentrated on several problems: The mechanism of processing of eukaryotic RNAs; the basic cellular mechanism involved in the transport of RNA from nucleus to cytoplasm; and the expression of ALU sequences in eukaryotes. Clinical research in the Section has focused on the mechanisms of bone formation in man, especially as these mechanisms relate to the pathophysiology of osteogenesis imperfecta and fibrodysplasia ossificans progressiva; the expression of human collagen genes; and the structure of the bone-specific alkaline phosphatase gene.

Over the past several years, this group has studied the pathway of expression leading to biosynthesis of the $tRNA_i^{met}$ molecule in human cells. These studies have provided insights into the organization of the tRNA gene family in man, and the pathways utilized in delivery of a mature tRNA into the cytoplasm of eukaryotic cells. This laboratory had previously shown that a naturally occurring $tRNA_i^{met}$ variant exhibited a defective phenotype in several *in vitro* systems. The group demonstrated that the natural variant gene contained a point mutation which resulted in the appearance of a primary gene transcript that was inefficiently processed. In addition, through the use of microinjection and micro-dissection methodology in the *X. laevis* oocyte system, the group demonstrated that the variant tRNA species was defective in its transport from the nucleus to the cytoplasm. This discovery led to the first description of the mechanism by which an RNA is transported from the nucleus of a eukaryotic cell. It was demonstrated, in fact, that tRNA species are transported by a saturable carrier-mediated mechanism. These investigators proposed that a ribosome-like element at the nuclear envelope was the actual "motor" utilized in this process. To fully explore the domain of the $tRNA_i^{met}$ molecule recognized by the transport system, thirty point mutations were generated in the human gene by *in vitro* mutagenesis utilizing hydroxylamine. Analysis of the resultant transport phenotypes yielded the unexpected finding that the tRNA molecule is exquisitely sensitive to mutation in its transport properties. The particularly sensitive areas include the t and D loops of the tRNA, the most highly conserved portions of the species. Another feature of this study was the demonstration that every mutation which yields a tRNA species defective in transport, also yields a pre-tRNA species which is inefficiently processed *in vivo*. This correlation led to the postulate that the processing enzymes might in some manner be playing a role in tRNA transport. As a result, the processing nucleases were purified from eukaryotic cells, representing the first characterization of this class of enzyme in eukaryotes. This group demonstrated that two enzymes, both endonucleases, process the primary transcript of the human $tRNA_i^{met}$ gene to a mature species. The first reaction involves cleavage of the 5' leader; the second, cleavage of the 3' trailer. Two enzymes have been purified from *X. laevis* oocytes and KB cells. The enzyme which processes the 3' trailer is a simple polypeptide of about 97,000. Its most striking

feature is that it will only cut the 5' processed primary transcript, establishing a cutting order within the pathway. The first cutting activity is that of the 5' nuclease. This enzyme has been purified to homogeneity and appears to be one of the most complex enzymes yet described in animal cells. It is composed of at least 14 different polypeptides ranging in MW from 20,000 to 32,000. It has the shape of a cylinder, composed of a stack of 4 rings. The entire structure appears to be necessary for tRNA processing. The precise relationship between its structure and enzymatic activity is under study. It is noteworthy that while this particle has been described in the literature over the past 15 years (having been isolated from organisms ranging from Drosophila to man), its function remained a mystery until this current work. The relationship between this particle and tRNA transport remains to be ascertained.

Work was begun this year to define the mechanism of mRNA nuclear transport, a fundamental eukaryotic process about which virtually no solid information exists. The system utilized has been the X. laevis oocyte, with the mRNA being the Herpes TK gene transcript. This product is efficiently synthesized and functional enzyme can be assayed within several hours of gene microinjection,a demonstration of the integrity of the entire expression pathway for this gene in the X. laevis oocyte. These workers developed new methodology to study mRNA translocation and have defined the intracellular distribution of RNA following gene transfer. The most striking finding is that mRNA remains intranuclear for about 90 minutes, at which time all RNA transcribed from the TK gene is suddenly and quite efficiently transported. Virtually no steady state levels of TK sequence accumulate in the nucleus after 90 min. The question to be answered next involved those structural features of a eukaryotic mRNA which perturb movement between compartments. The role of translation punctuation signals in mRNA movement will be explored utilizing appropriate in vitro constructs.

M. Zasloff and S. Adenyi-Jones have continued their study of the Alu sequence family. These sequences comprise around 3-6% of the vertebrate genome, present in about 300,000 copies. These investigators have demonstrated that one such sequence, the murine E1 sequence, is processed and transported from nucleus to cytoplasm. The particular sequence studied lies antisense to a murine gene encoding alpha-fetoprotein (AFP). The processing reaction involving the Alu primary transcript appears to be endonucleolytic, releasing the 3' trailer. The "core" Alu RNA generated is transported upon processing into the cytoplasm. This group has shown that this species is present in highest abundance in murine fetal liver, the tissue in which AFP is most actively expressed. This result was unexpected since current notions would assign an inhibitory role to this natural antisense RNA. The Alu sequence RNA, in addition, was shown to be associated with a specific polypeptide of about 63,000 MW, a polypeptide identified through the use of an autoimmune antiserum from a patient with lupus. This polypeptide appeared to be associated with the primary transcript of the Alu sequence in its nuclear phase, and remained associated after processing and transport to the cytoplasm. The affinity-purified antibody further identified a protein of similar size in KB cells. Associated with this polypeptide in the human cells was a small RNA of about 80 nt. It may be that this RNA represents the human analogue of the E1 Alu family. The results suggest that despite nucleic acid sequence divergence, the Alu sequence may be functionally conserved between vertebrates. Its role in gene expression is under study in several systems.

In clinically-related studies, the pathophysiology of hypophosphatasia was investigated at a molecular genetic level. The initial approach has been to isolate the cDNA and gene encoding human bone-specific alkaline phosphatase (AP). Consequently, the human enzyme was purified to homogeneity from liver, and partial sequence information obtained. Appropriate synthetic oligonucleotides were generated, and cDNA libraries are of being screened for potential AP clones. With these probes at hand, the basic defect in this disorder of ossification can be explored.

Studies on the pathophysiology of osteogenesis imperfecta (OI) by J. Marini continue. After considerable effort, the transcription units for alpha-2 type I collagen were determined in skin fibroblasts and in osteoblasts of avian and human origin. In both cases, the mRNAs do not undergo alternative splicing or variations in promotor usage in bone as compared with skin fibroblastic cells. This result suggests that other mechanisms must operate to explain the considerably greater abundance of collagen-specific mRNA in the osteoblast, including tissue-specific enhancers, 3' splicing alternatives, or differential stability.

To pursue the pathophysiology of OI, techniques for identification of a mutation in the collagen polypeptide genes were employed. These included mRNA/DNA mismatch detection, polypeptide synthesis in culture, thermal stability of secreted procollagen, and restriction length polymorphism. It is expected that as mutations are identified, they will be used to extend our understanding of the role of collagen structure in bone development.

In another OI study, attempts were made to determine if a defect in the known growth-promoting hormones is correlated with growth failure in this disorder. It appears that growth failure is seen variably in affected individuals, unrelated to the severity of bone fragility. The current studies involve evaluation of growth hormone (GH) secretion as well as IGF-I and IGF-II levels, both in the steady state and provoked. Initial studies suggest that children with severe OI may have extremely low levels of circulating IGF-I, suggesting a role for this hormone in the process. The apparent decrease in GH secretion in some affected patients has prompted the use of clonidine as a stimulant of endogenous secretion.

Fibrodysplasia ossificans progressiva (FOP) is a rare inherited disorder of heterotopic ossification characterized by relentless appearance of mature enchondral bone within and around skeletal muscle. With respect to pathophysiology, it was demonstrated this past year that all patients with FOP possess a circulating prostanoid which cross-reacts with PGE2 by radioimmunoassay. This prostanoid appears to be present in patients' plasma at between 10-100 fold higher levels than in unaffected individuals. The exact nature of the compound is being determined at the present time through mass spectroscopic analysis. It may represent a stable, well described metabolite. If so, these results suggest that prostanoid synthesis in FOP patients is markedly accelerated. However, if the compound represents a new prostanoid, its structure may suggest that it is a bone inducer. Studies of one patient with active bone formation have revealed that this prostanoid is not secreted by the bone lesion itself.

Finally, this group identified a new disorder of heterotopic ossification in children during the past year. It appears to represent a disorder of dermal differentiation, and follows a dramatically different natural history than FOP.

The section headed by J. Sidbury continues its studies on the treatment of the glycogen storage diseases, mineral metabolism in man, and the pathophysiology and treatment of various forms of childhood obesity. They have shown that glycogen storage disorders can be effectively treated by oral administration of cornstarch, an easy approach which yields predictable blood glucose levels with little fluctuation, replacing the complex and often fatal treatments previously attempted in these diseases. The group continues to study means of optimizing therapy, including a study of the therapeutic efficacy of several different naturally occurring vegetable starches. With rare exceptions, corn starch was found to be the most effective therapy in terms of sustaining a normal blood glucose level over a 6-hour period in patients with glucose-6-phosphatase deficiency. Studies have been undertaken to determine if the serum amylase can be used to detect heterogeneity, and to learn whether very young children (<4) have sufficient pancreatic amylase to be candidates for starch therapy.

The liver glucose production rates have been determined in four patients with type I glycogen storage disease and in one with type III, verifying the production of glucose by individuals with deficient glucose 6-phosphatase activity. The rate of production in those with total absence of activity can be distinguished from those with partial deficiency.

In studies on morbid obesity, Sidbury et al. made an assessment of the potential efficacy of nalmefene, a widely touted third generation compound, in the control of appetite in the Prader-Willi syndrome. The drug was found to be without effect in this situation.

Caddell and Sidbury have begun a study of the consequences of magnesium deficiency in the human neonate, with parallel studies in an animal model (the weanling rat fed varying levels of dietary magnesium). One hypothesis suggests that magnesium deficiency could be linked to the "Sudden Infant Death" syndrome, and Caddell's investigation includes a retrospective analysis of data from 200 premature neonates with apnea neonatorum to learn whether magnesium supplementation is beneficial. Using the weanling rat as a model, studies have also been concerned with catecholamine release in magnesium deficiency, the interaction of magnesium and calcium in furosemide-treated rats, and light and electron microscopic studies of the heart and lung in acute magnesium deficiency.

A study of infants with idiopathic apnea neonatorum was completed in which these patients were or were not treated with magnesium. Those treated had fewer recurrences. Mice made magnesium-deficient and subjected to an audiogenic or strychnine seizure developed lung pathology similar to that found in hyaline membrane disease, but normal mice did not show such pathology.

The section led by A. Mukherjee conducts research to understand (1) the physiological role and genetic regulation of endogenous phospholipase A2 inhibitory proteins such as uteroglobin and lipocortins, and (2) the mechanism of intrauterine growth retardation and the genetic predisposing factors in Fetal Alcohol syndrome (FAS).

During the past year, these workers demonstrated that uteroglobin (Utg), a steroid-dependent, low molecular weight secretory protein in the rabbit, is a potent inhibitor of phospholipase A2 (PLA2) activity. Utg has been found to inhibit PLA2 both in cultured cells as well as in a cell-free system. Since PLA2 activity is required to generate arachidonate, the substrate for prostaglandin (PG) synthesis, experiments were carried out to delineate whether Utg inhibited cellular prostaglandin levels. To study this activity and the regulation of the Utg gene expression, cell lines from all organs of the rabbit wherein Utg is found have been established by transformation with an SV40-tsA mutant. Two such cell lines derived from the adult rabbit endometrium (RBE-7 and H5DC), and one cell line derived from young rabbit endometrium (YRE-1), have so far been characterized. At 39.4°C, when stimulated with progesterone (10^{-9} M) after 4 days of estradiol (10^{-9} M) priming, an increased rate of transcription as well as translation of the Utg gene occurs. These cells, when stimulated with estradiol or phorbol ester, were found to have a high PLA2 activity and consequently, a high level of prostanoids (PGE2, PGF2α, 6KPGF1 α, and thromboxane B2). When the cells were further treated with exogenous Utg (20 μM), a 78% reduction in PLA2 activity occurred with an 85% inhibition in prostanoid levels as compared to control values. To determine whether endogenous Utg production by these cells would reduce PLA2 activity, the phorbol ester pre-treated cells were exposed to progesterone (10^{-9} M) for 24 hours, when Utg production was detected. Significant reduction in PLA2 activity, as well as lowered prostanoid levels (as measured by HPLC and RIA), was observed. This reduction of PLA2 activity and/or prostanoid levels was not observed when either cyclohexamide, a protein synthesis inhibitor, or a monospecific antibody to Utg was added following progesterone treatment of these cells. The YRE-1 cell line behaved similarly to RBE-7 and H5DC cells when the same experiments were repeated. Preliminary *in vivo* experiments appear to confirm the *in vitro* results.

Last year, preliminary results concerning the detection of a human protein similar to rabbit Utg were presented. Further experiments have confirmed that there is a low molecular weight secretory protein present in the human tracheobroncheal tract as well as the endometrial epithelium during the midluteal phase of the cycle. In collaboration with B. Cowan (University of Mississippi) and H. Zacur (Johns Hopkins Hospital), this group studied endometrial washings and endometrial tissues from 48 women at different stages of the menstrual cycle. It was confirmed that the Utg-like protein appears in the midluteal phase of the human endometrium. Additional studies are now in progress to determine whether there is any correlation between the presence or absence of this protein and habitual abortion. Two human endometrial cell lines have been established using SV40 transformation. These cells are now being characterized for the production of the Utg-like protein. If enough protein is produced by these cells, an antibody will be raised and production of a cDNA clone of the human gene will be attempted.

To delineate the active site of the Utg molecule involved in PLA1 inhibition, site-directed mutagenesis of the Utg gene and its expression in a bacterial vector have been undertaken this year. These mutated proteins will be tested for PLA1 inhibiting activity. Studies have also begun on the expression of the Utg gene in *E. coli*. A full-length cDNA containing the entire pre-Utg coding region was obtained from D. Bullock (Baylor College of Medicine), and from this cDNA, a 490 base pair Pst-I fragment was prepared. This fragment contains the

entire mature coding region, starting with the Gly-1 codon. This fragment was subcloned in the Pst-I site of the expression vector pKK233-3,and the orientation of the insert was checked by restriction digestion; one of the positive clones (pLE-101) was tested for protein expression. In this expression system, the cDNA insert is under the control of the synthetic "trc" promotor, which in turn is controlled by the lac repressor protein when a lac I^q E. coli host is used. Two different lac I^q hosts were employed, JM-105 and JM-109. After transformation with pLE-101 and colony purification, single Amp^r colonies were grown in LB-Amp medium up to 0.7 OD_{650} and induced with isopropyl-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. After 1 hour, bacteria were harvested and Utg production was tested by RIA on aliquots of bacterial lysates. Uninduced controls were run in parallel. The level of Utg production was 1.25 µg/gm of cells for JM-105, and about 5 µg/gm of cells for JM-109. Since the purpose of these experiments was to obtain large amounts of purified recombinant Utg, these levels of expression were too low. Therefore, two new expression systems based on the lambda-PL promotor will be tested: pAS_1 and pRC23. These vectors contain synthetic strong ribosome binding sites that assure efficient translation of the insert upon induction by temperature shift. Moreover, protease deficient (lon^-) E. coli strains will be used to minimize protein loss due to proteolysis.

In Mukherje's second area of interest, the role of genetic predisposing factors is studied whith respect to ethanol toxicity. During the past year, these workers studied a transketolase abnormality in cultured fibroblasts from a diabetic patient who developed Wernicke's encephalopathy when treated with tolazamide, a hypoglycemic agent. Additionally, three diabetic kindreds, without any history of Wernicke's encephalopathy, and four normal controls were studied. It was found that the patient with tolazamide-induced Wernicke's encephalopathy and one of the three diabetic kindreds had an abnormal K_m of the transketolase for TPP (thiamine pyrophosphate) similar to the K_m's found previously in post-alcoholic Wernicke-Korsakoff syndrome. These data suggest that the transketolase abnormality is prevalent in the population, and the individuals with this abnormality may be predisposed to developing thiamine deficiency syndromes either by exposure to drugs that increase thiamine utilization or by ethanol abuse. To investigate whether or not this enzyme abnormality is associated with the fetal alcohol syndrome (FAS), two cultured amniotic fluid cell lines were obtained from neomates diagnosed to have FAS by several criteria. Three control amniotic fluid cell lines from normal pregnancies were also investigated. Preliminary results suggest that both amniotic cell lines from FAS pregnancies have an abnormal transketolase enzyme as suggested by the determination of the K_m of this enzyme for TPP as compared to control values. Mukherjee et al. will investigate the cells from additional FAS, and normal control pregnancies as well as cells derived from pregnancies wherein the mother had a history of alcohol abuse, but the baby was unaffected. In a related study, this group has investigated the transketolase enzyme kinetics in fibroblasts from ten members representing three generations of an Amish family. The results show an autosomal recessive type of inheritance of the abnormality, as previously suggested by V. McKusick.

The section headed by William Gahl investigates the clinical and basic research aspects of inborn errors of metabolism in man. During the past year, the cause of a rare lysosomal storage disease was documented. Salla disease, a Finnish disorder characterized by psychomotor retardation and lysosomal accumulation of free sialic acid, was shown to be caused by a deficiency of normal free sialic

acid transport across the lysosomal membrane. This result identifies Salla disease as the second storage disorder due to impaired transport of a small molecule out of lysosomes, and sialic acid the first monosaccharide for which a carrier-mediated transport system within the lysosomal membrane has been described. Three years ago, the section reported that cystinosis was the first lysosomal storage disorder due to a transport deficiency (for cystine); these two discoveries have created a new field in the area of human metabolism, with the identification of similar disorders likely to follow.

Work by the section has employed the technique of counter-transport to demonstrate the existence of a new lysosomal transport system for tyrosine and other neutral amino acids in cultured rat thyroid cells. The carrier system resembles the L system described in plasma membranes. Rat thyroid cells were also shown to possess a lysosomal cystine carrier closely resembling that of human leucocyte and fibroblast lysosomes. The rat carrier system exhibited a half-life of approximately 24 hours when cells were cultured in the presence of the protein synthesis inhibitor, cycloheximide. Total genomic DNA from the rat cells will be used to transfect human cystinotic fibroblasts, followed by a selection technique that kills the mutant, but not the "cured" cells.

Cultured fibroblasts from patients with mucolipidosis II (I-cell disease), which store free cystine in their lysosomes, were shown to lack normal amounts of lysosomal cystine transporting capacity. In similar experiments, cells from a patient with intermediate (late-onset) cystinosis exhibited no lysosomal cystine egress, just as in nephropathic cystinosis cells. However, leucocytes from a patient with benign cystinosis, who had no renal disease and limited cystine storage, displayed a significant amount of residual lysosomal cystine-carrying capacity. Thus, the severity of clinical disease in cystinosis may be related to the degree of residual cystine carrier as well as other genetic and environmental factors.

The section has vigorously pursued a putative defect in glycosaminoglycan sulfation in the Lowe (oculocerebrorenal) syndrome, demonstrating normal synthesis and sulfation of proteoglycans in this X-linked disorder of mental retardation, congenital cataracts, and renal Fanconi syndrome. Both cultured fibroblasts and muscle cells were examined in these studies.

In clinical studies, Gahl et al. have treated a large group of nephropathic cystinosis patients in the United States and Canada (approximately 25) with the cystine-depleting agent, cysteamine, for the past 8 years. A significant improvement in growth and a retardation of renal deterioration have been appreciated. At the same time, serious post-renal transplant complications of cystinosis have been documented, including diabetes mellitus, cerebral atrophy and calcifications, and ocular abnormalities such as blindness, posterior synechiae and corneal erosions. These findings have prompted the initiation of cystemaine therapy for post-transplant cystinosis patients and the performance of a corneal transplant in one 12-year-old boy with debilitating corneal erosions.

Other ongoing therapeutic trials involve oral L-carnitine repletion for carnitine-deficient patients with the renal Fanconi syndrome, and betaine therapy for pyridoxine-nonresponsive homocystinuric patients. In addition, a 31-year-old man with hypermethioninemia was diagnosed as having hepatic

methionine adenosyltransferase deficiency. Prior to this, the enzyme deficiency was described only in young children. Consequently, this individual adds 25 years of natural history to our current knowledge of the disorder in man. He also represents an example of the type of rare disorder this section continues to investigate to gain insight into the causes and effects of metabolic deficiencies in man.

The studies in the section led by Janice Chou concern regulation of gene expression during normal and abnormal differentiation processes. In the past year, studies on the expression of the alpha-fetoprotein (AFP) gene in temperature-sensitive (ts) fetal liver cells were continued. It was demonstrated that transformed fetal hepatocytes synthesize a variant AFP of 65K which is encoded by an mRNA of 16S. However, differentiated fetal hepatocytes synthesize two AFPs of 73K and 69K, which are encoded by an mRNA of 20S. Hybridization experiments demonstrate that the two mRNAs differ in sequences at the 5' end. Nuclease S1 mapping experiments showed that the 16S mRNA lacks the first six exons (Z, A, B, C, D, E) which are normally present in the 20S RNA. Since there is only AFP gene per haploid genome in rat, the two AFP mRNA species may be generated by the use of different promoters.

In studies on the hormonal regulation of gene expression, Chou and her colleagues found that glucocorticoid hormone is a potent agent that induces fetal maturation. They studied regulation of expression of specific fetal and adult genes in the ts fetal hepatocyte line established in this laboratory. At 40°C, these cells exhibit a differentiated phenotype; glucocorticoid hormone suppressed expression of the fetal gene, AFP, but induced expression of the TAT gene. (TAT is an enzyme that appears only postnatally.) Thus, fetal liver cells mature into adult hepatocytes in the presence of glucocorticoid hormone.

To ascertain how closely the ts hepatocyte systems resemble primary liver cells, Chou's group studied the ability of the adult hepatocytes to express a large number of liver-specific functions. In addition to expressing genes encoding AFP, albumin, and transferrin, they found that these cells express genes encoding TAT, phosphoenolpyruvate carboxykinase (PEPCK), and fibrinogen. As expected, expression of these genes is ts and expressed mainly at the nonpermissive temperature of 40°C. At 40°C, these cells exhibit a normal differentiated phenotype. Most importantly, expression of all of these genes is dependent upon the presence of glucocorticoid hormone. In collaboration with Dr. G. Yeoh of Western Australia, Chou et al. studied the expression of the TAT gene in detail. They found that transformed hepatocytes synthesize low levels of TAT, but differentiated cells synthesize greatly increased amounts of this enzyme. Glucocorticoid hormone was absolutely required to sustain TAT expression. Differentiated hepatocytes synthesized no detectable levels of TAT in the absence of glucocorticoid. cAMP enhances the induction by glucocorticoid, but cannot replace this steroid hormone. TAT synthesis was not detectable in differentiated hepatocytes in the presence of cAMP alone. Furthermore, these workers demonstrated that the change in TAT synthesis paralleled the change in TAT mRNA levels.

Studies on the expression of the human placental alkaline phosphatase (PAP) gene at the molecular level were continued. This group found that different PAP mRNAs were expressed in choriocarcinoma and HeLa cells as compared to human placenta. Choriocarcinoma cells are malignant trophoblasts which produce PAP eutopically, whereas HeLa cells produce this phosphatase ectopically. PAP mRNA

of placenta migrated as a 2.7-kb band, whereas PAP mRNAs from choriocarcinoma and HeLa cells migrated as 2.4-kb and 2.6-kb bands, respectively. Sodium butyrate induced the biosynthesis of PAP and increased the PAP mRNA levels in both types of cells. HeLA PAP synthesis was induced also by prednisolone. The apparent molecular weight of the PAP monomer in both types of cells, in the presence or absence of inducers, was similar. In choriocarcinoma cells, sodium butyrate increased the levels of the 2.4-kb PAP mRNA. However, in HeLa cells, prednisolone induced the expression of the 2.7-kb PAP mRNA, whereas butyrate induced the expression of two mRNAs for PAP, the 2.7-kb band and a fast migrating band at 2.5-kb. Analysis of the mRNA and genomic structure of PAP should yield information as to its polymorphism.

Studies on the expression of the pregnancy-specific β-glycoprotein (PSβ-G) gene at the molecular level have been extended over the past year. Genes encoding PSβ-G were cloned. Efficient isolation of these genes was accomplished by probing a phage lambda gt11 expression library with antibodies to PSβ-G. The identity of the cDNA clones was confirmed by comparing the predicted amino acid sequences to sequences determined from fragments of placental PSβ-G. These probes will be used to examine molecular mechanisms regulating PSβ-G synthesis in placental cells and in fibroblasts.

Human Genetics Branch

Chief, *Michael A. Zasloff, M.D., Ph.D.

Senior Staff

*Janice Y. Chou, Ph.D.
*William A. Gahl, M.D., Ph.D.
*Anil B. Mukherjee, M.D., Ph.D.
*James B. Sidbury, M.D., Ph.D.
Jean Butler, Ph.D.
Fellows

Lawrence Charnas, M.D.
Andrew Dancis, M.D.
Kenneth Huttner, M.D., Ph.D.
Joan C. Marini, M.D., Ph.D.
Wesley Picolo, M.D.
Janet A. Tobian, Ph.D.
Yu-Jui Yvonne Wan, Ph.D.

Visiting Program

Samuel Adeniyi-Jones, M.D., Ph.D.
Juan Bernar, Ph.D.
Gregory Harper, Ph.D.
Takahiro Kikukawa, Ph.D.
Juan Jimenez-Molina, M.D.
Lucio Miele, M.D.
Pilar de la Pena, Ph.D.
Shuichiro Watanabe, M.D.

Guest Researhers

Megan Adamson, M.D.
Joan L. Caddell, M.D.
Ramsasubbareddy Dhanireddy, M.D.
Kathy Grange, M.D.
Johanna G. Koster, Ph.D.
Sondra Levin, M.D.
Wu Bo Li, Ph.D.
Jian Liu, M.D.
Giuseppi Martini, M.D.
Martin Renlund, M.D., Ph.D.
Raili Seppala, Ph.D.
Frank Tietze, Ph.D.

Bibliography

Caddell JL, Kupiecki R, Proxmire DL, Satoh P, Hutchinson B. Plasma catecholamines in acute magnesium deficiency in weanling rats. J Nutr 1986;116:1896-1901.

Castano JH, Ornberg R, Tobian JA, Zasloff M. Eukaryotic pre-tRNA 5' processing nuclease: copurification with a complex cylindrical particle. Cell 1986;46:377-87.

Chou JY, Savitz AJ. Alpha-fetoprotein synthesis in transformed fetal rat liver cells. Biochem Biophys Res Commun 1986;135:844-51.

Cowan BD, North DH, Whitworth NS, Fujita R, Shumacher EK, Mukherjee AB. Identification of a uteroglobin-like antigen in human uterine washings. Fertil Steril 1986;45:820-3.

Foster CM, Levin S, Levine M, Mukherjee A, Costa J, Eanes D, Triche J, Zasloff M. Limited dermal ossification: clinical features and natural history. J Pediatr 1986;109:71-6.

Gahl WA. Cystinosis, coming of age. Adv. Pediatr 1986;33:95-126.

Gahl WA, Adamson M, Kaiser-Kupfer ML, Ludwig IH, O'Connell HJ, Cohen W. Biochemical phenotyping of a single sibship with both cystinosis and fabry disease. J Inherited Metab Dis 1985;8:127-31.

Gahl WA, Dorfmann MI, Karson EM, Jandsberger EJ, Fabco SE, Schulman JD. Chronic biopsy in the parental diagnosis of nephropathic cystinosis. In: Fraccoro M, et al., eds. First trimester fetal diagnosis. Berlin, Heidelberg: Springer-Verlag, 1985;260-2.

Gahl WA, Schneider JA, Thoene JG, Chesney R. Course of nephropathic cystinosis after age 10 years. J Pediatr 1986;109:605-8.

Levin SW, Butler J DeB, Schumacher UK, Wrightman PD, Mukherjee AB. Uteroglobin inhibits phospholipase A_2 activity. Life Sci 1986;38:1813-9.

Levin SW, Roecklein BA, Mukherjee AB. Intrauterine growth retardation caused by dietary biotin and thiamine deficiency in the rat. Res Exp Med 1985;185:375-81.

Ovitt CE, Strauss AW, Alpers DH, Chou JY, Boime I. Expression of different-sized placental alkaline phosphatase mRNAs in placenta and choriocarcinoma cells. Proc Natl Acad Sci 1986;83:3781-5.

Renlund M, Tietze F, Gahl WA. Defective sialic acid egress from isolated fibroblast lysosomes of patients with salla disease. Science 1986;232:759-69.

Schulman JD, Tietze F, Gahl WA. Cystinosis. In: Bickel H, Wachtel U, eds. Recent progress in the understanding, recognition and management of inherited diseases of amino acid metabolism. Stuttgart: Verlag-Springer, 1985;256-64.

Sidbury JB, Chen Y-T, Roe CR. The role of raw starches on therapy of type I glycogenesis. Arch Intern Med 1986;146:370-3.

Sun IL, Crane FL, Chou JY. Modification of transmembrane electron transport activity in plasma membranes of Simian virus 40 transformed pineal cells. Biochem Biophys Acta 1986;886:327-36.

Tietze F, Rome LH, Butler J DeB, Harper GS, Gahl WA. Impaired clearance of free cystine from lysosome-enriched granular fractions of I-cell-disease fibroblasts. Biochem J 1986;237:9-15.

Tobian JA, Drinkard L, Zasloff MA. tRNA nuclear transport: defining the critical regions of human $tRNA^{met}$ by point mutagenesis. Cell 1985;43:415-22.

Wittwer CT, Gahl WA, Butler JD, Zatz M, Thoene JG. Metabolism of pantethine in cystinosis. J Clin Invest 1985;76:1665-72.

Cell Biology and Metabolism Branch--
Richard D. Klausner, M.D., Chief

The structure and organization of the Cell Biology and Metabolism Branch became more defined in this first full year of operation, and a large addition to Building 18 was completed, providing new laboratory space, equipment space, autoclave room and small animal holding room.

Research in this Branch is directed toward clarifying developmental aspects of intracellular structure and function. Various methods are being utilized to study receptor biosynthesis, dynamics, regulation, and degradation, using the human transferrin receptor, the T-cell antigen receptor, and the interleukin-2 receptor as models. (Receptors are a complex set of molecules that mediate virtually all aspects of interactions between the cell and its environment. They mediate cell-cell communication via hormones and neurotransmitters, invasion by viruses, uptake of nutrients, regulation of growth and differentiation, and recognition of specific molecules.) The Branch also conducts clinical research on the fundamental mechanisms and treatment of patients with genetic disorders of iron metabolism (hemochromatosis) to learn how the intracellular traffic of iron is normally controlled, and at what level iron metabolism is abnormally regulated in such patients. In another area of interest, the Branch examines mechanisms by which intracellular architecture is maintained, providing the basis for the function and dynamics of cellular organelles.

Structure and Function of the T Cell Antigen Receptor

This work, led by R. Klausner, is focused on receptor structure, biochemistry of receptor activation, receptor function in immune disease, and receptor assembly. The description of the T cell antigen receptor complex has been extended with the recognition of a seventh protein. Previously this group had described and characterized six different proteins which, because one is a homodimer, form a complex of seven peptide chains. Each was purified and polyclonal antibodies were raised against all chains. The recently published nucleotide sequence of a murine cDNA clone homologous to the human T2 delta chain was used to design synthetic peptides which were then utilized to raise an extremely specific anti-murine δ chain antiserum. These immunological tools have made feasible studies on the biosynthesis, assembly, degradation, distribution and biochemistry of the receptor in any T cell, and alleviated the previous constraint of having to utilize only those T cell clones or hybridomas for which anti-clonotypic antibodies were available. In addition, cross-reactivity between human and murine chains permitted the group to assign correct homologies. This latter approach allowed the group to identify the gp26 glycoprotein as the δ chain of the murine complex. The development of anti-ξ chain antibodies allowed the identification of the human homologue of this receptor component, following upon the original discovery of this chain in this laboratory.

Studies on the biochemistry of T cell antigen receptor activation progressed dramatically over the past year. These workers recognized that antigens and mitogens induce the simultaneous activation of two distinct phosphorylation pathways. One involves the activation of protein kinase C via the enhanced metabolism of phosphorylated phosphatidyl inositols. This leads to the serine phosphorylation of the γ chain of the complex. In addition, a previously unrecognized component of the complex is phosphorylated on tyrosine. This

chain is a 21 kd endoglycosaminidase F-resistant protein (p21), with a more acidic pI than the γ chain, that exists in the complex as a disulfide-linked dimer. (It is still unclear whether it is a homo-or heterodimer.) The finding of a tyrosine kinase coupled to the T cell antigen receptor places this receptor in that small group of cellular receptors involved in growth and differentiation, all of which are coupled to tyrosine phosphorylation events. Both the tyrosine and serine phosphorylations represent true de novo addition of phosphates and not phosphate exchange. Although both phosphorylation pathways are activated upon ligand binding, the tyrosine phosphorylation does not require the presence of protein kinase C. Cyclic AMP has long been known to block the immune response. Klausner et al. have demonstrated that cAMP uncouples the receptor from both phosphorylation pathways. Protein kinase C antagonizes the effects of cAMP on tyrosine phosphorylation but not phosphatidyl inositol metabolism, thus further complicating our understanding of the interactions between these kinases in the activation of this receptor. These workers recently succeeded in reconstituting tyrosine phosphorylation of the receptor in isolated membranes. This promises to allow them further to characterize and dissect the biochemical events observed in intact cells. The implication of a phosphatidyl inositol kinase in the coupling of this receptor to the activation of protein kinase C led the group to attempt to characterize and purify this important cellular enzyme. The characterization of the enzyme has been extensive, and much progress has been made on its complete purification. Currently, the enzyme is 1000-2000 fold purified.

Characterization of the phosphorylation events in cloned T cells or antigen-specific T cell hybridomas has been extended to normal T cells, including human T lymphocytes. In addition, two genetically distinct but clinically identical autoimmune/lymphoproliferative diseases of mice have been studied. (These provide useful models for human autoimmune diseases.) Because of the central role of the T cell antigen receptor in T cell activation, the possibility that these animals have abnormal receptors was tested. Both of these diseases display the same unusual features of receptor activation: 1) p21 is spontaneously phosphorylated on tyrosine even in the absence of any added ligand; 2) this takes place in the absence of any detectable serine phosphorylation; and 3) mitogen fails to activate these receptors. These results offer us the first clue as to possible biochemical lesions responsible for autoimmune diseases.

The question of how the cell assembles a 7-9 chain membrane complex with a predictable and unique stoichiometry is a fundamental one in cell biology. This question has been addressed by examining the kinetics and pattern of assembly of the T cell antigen receptor after its biosynthesis. Assembly occurs rapidly after biosynthesis, and some of the chains are synthesized in great excess, with 95-98% of the newly synthesized molecules being degraded with a half-life of about 30 minutes. Only completely assembled chains survive with a half-life of between 12 and 20 hours. The degradation of unassembled or incompletely assembled chains appears to occur in lysosomes. This has suggested a general model of assembly whereby the state of assembly (or lack thereof) determines the intracellular routing of integral membrane proteins.

Interleukin-2 Receptor: Structure, Function and Regulation

W. J. Leonard's group is studying the interleukin-2 (IL-2) receptor which is responsible for the proliferation of T cells during the immune response. Understanding how its expression is regulated and how this receptor functions are

central problems in immunology. In addition, the IL-2 receptor has been implicated in the abnormal proliferation of leukemic T cells. The work of this group can be divided into two areas: Transcriptional control elements, and structure of the high affinity receptor. The regulatory regions located 5' to the structural gene that encodes this receptor have been cloned and partially characterized. When constructs consisting of these flanking regions are fused to the structural gene for bacterial chloramphenicol acetyl transferase, several important conclusions are apparent. First, the regulatory sequences of this gene are highly sensitive to one or more gene products of the human T cell lymphocytotropic I virus (HTLV-I) and expression of the hybrid genes is much greater in HTLV-I infected cells. (This virus is the cause of adult T-cell leukemia.) The hybrid genes are responsive to treatment of the host cells with phorbol diesters (tumor promotors) when the genes are introduced in JURKAT cells. This cell is not infected with HTLV-I, and the expression of its IL-2 receptor is dependent upon treatment with phorbol esters. Finally, in HTLV-I infected cells a dramatic rise in expression of the hybrid genes is seen when a far upstream 5' flanking region is removed. This strongly points to a negative regulatory element within this gene. Once interesting regulatory sequences are identified, the next step will be the identification of specific binding proteins that interact with these sequences. Preliminary progress has been made in this effort, with the identification of proteins which bind to specific regions of the 5' end of this gene.

How the IL-2 receptor functions remains a mystery. The nature of the signals generated by the binding of the ligand to the receptor are, as yet, undefined. One clue involves the observation that there are two classes of IL-2 receptors as defined by ligand binding. One class, representing 90-95% of all binding sites, displays affinities in the nanomolar range, while the rest display binding affinities in the picomolar range. The minority high affinity receptors are entirely responsible for both the endocytosis and activity of the ligand. Thus a critical question is what defines the biochemistry of this high affinity receptor. Studies using covalent cross linking of radiolabeled IL-2 to intact cells have begun to illuminate this problem. When IL-2 was bound to T cells under high affinity conditions and cross-linked, it was shown to interact with two proteins - the known 55 kilodalton receptor chain, and a previously unidentified 70 kilodalton chain. Cross-linking of ligand to low affinity sites showed that the IL-2 in that situation is only interacting with the 55 kilodalton receptor glycoprotein. Thus the high affinity receptor is likely composed of at least two subunits.

Regulation of Intracellular Iron Metabolism

During the past year, the major project of this group, led by R. Klausner, has been to clone molecularly the gene for the human ferritin heavy chain. Previous work from this lab has pointed to ferritin as the regulator of intracellular iron distribution. This mechanism, in turn, was regulated by iron itself which determines the level of ferritin within the cell. In order to develop the tools to study the underlying mechanisms involved, the full gene encoding a ferritin chain is needed. Previous work had demonstrated that many copies of both ferritin heavy (H) and light (L) chain genes are scattered throughout the human genome. Many of these are likely to be processed pseudogenes, and finding an expressed gene thus required a screening strategy to avoid pseudogenes. Such a strategy was designed and a full length copy of the human H chain ferritin gene isolated. This was sequenced and shown to contain a large (~260

base) 5' untranslated region and three introns. The entire length of the gene is about 3 kb. The gene has been introduced into mouse fibroblasts where it has been shown to be expressed. The reconstituted gene functions normally in the murine cells. It is highly regulated by iron, assembles into normal ferritin spheres, and co-assembles with mouse ferritin. The functional gene is localized to chromosome 11. Interestingly, preliminary evidence from in situ hybridization suggests the possibility of a chromosomal duplication event, placing a copy of this gene at the HLA locus of chromosome 6. This is the predicted localization of the gene believed to be defective in hereditary hemochromatosis. The expression of the cloned gene from human chromosome 11 in murine cells is allowing the identification of the nucleotide sequences involved in iron regulation.

Molecular Aspects of the Regulation of the Human Transferrin Receptor

The central role that the transferrin receptor plays in cellular iron metabolism has led the work of this group, led by J. B. Harford, to focus on the transcriptional control elements of the gene for the human receptor. The 5' region of the human gene was cloned and transcriptional elements were identified by constructing hybrid genes in which putative regulatory elements are fused to the structural gene for the bacterial enzyme chloramphenicol acetyl transferase (CAT). Such constructs have led to a number of conclusions. Two distinct elements are required in tandem to yield high levels of gene expression. One exists 5' to the transcription initiation site and the other resides in the first intron, approximately 1000 base-pairs downstream of the transcription initiation site. Deletion analysis, linker-scanner mutagenesis and sequencing have begun to elucidate the fine structure of these regions. The upstream region consists of two elements; one is located between bases -78 and -62 and contains a sequence homologous to the adenovirus Ela enhancer element and the other, located between -62 and -33, contains SP1 protein binding site sequences. Each contributes to the function of this region. The downstream site has been localized to less than 30 base pairs, and contains another homologue to the adenovirus enhancer element. This 3' control region cannot function in the reverse orientation. The identification of these sequences will allow the characterization of specific DNA binding proteins and of the regions required for iron regulation of the expression of this gene.

Membrane Traffic and Organelle Biogenesis

This group, led by I. V. Sandoval, proeviously developed monoclonal antibodies directed againstthe integral membrane proteins of various intracellular organelles. These antibodies have provided extremely useful tools for examining the relationships between different organelles, the trafficking between organelles, and the biogenesis of organelles. During the past year most effort was concentrated on a set of integral membrane proteins of the lysosomes. Biosynthetic pulse-labeling studies were used to examine the post-translational processing of these proteins. All are glycosylated and contain N-linked carbohydrate chains complexed with sialic acid. This finding demonstrates that these proteins are efficiently transported through the entire Golgi system and reach the trans-Golgi before being targeted to lysosomes. All lysosomal integral membrane proteins (LIMP) are transported through the Golgi at a similar rate but they are retained in the trans-Golgi for variable periods of time. This group has also developed an assay to monitor the transport of LIMPs from the Golgi to the lysosomes. This assay makes use of the very different buoyant

densities of the Golgi and lysosomes, and has allowed a clear demonstration of the rapid and efficient delivery of these proteins to lysosomes. Removal of N-linked carbohydrate chains has no effect on the transport of these proteins, but dramatically destabilizes the LIMPs in the lysosomes. These tools, and the information generated through their use, have created the possibility of examining the molecular mechanisms of protein sorting to lysosomes. This goal is now being approached, beginning with the molecular cloning of the genes encoding these lysosomal proteins.

Cell Biology and Metabolism Branch

Chief, *Richard D. Klausner, M.D.

Senior Staff

Joe B. Harford, Ph.D.

Fellows

Warren J. Leonard, M.D.
Myra A. Lipes, M.D.
Jennifer Lippincott-Schwartz, Ph.D.
John J. O'Shea, M.D.
Tracey A. Rouault, M.D.
Lawrence E. Samelson, M.D.
Michael Sharon, M.D.
Allan M. Weissman, M.D.

Visiting Program

Michael Baniyash, Ph.D.
Juan S. Bonifacino, Ph.D.
Bruno Di Jeso, M.D.
Yasuhiro Minami, M.D.
Ignacio V. Sandoval, M.D., Ph.D.

Guest Researchers

Javier Barriocanal, Ph.D.
John L. Casey, Ph.D.
Wright Caughman, M.D.
Sharon Cross, Ph.D.
Matthias Hentze, M.D.

Bibliography

Bonifacino JS, Perez P, Klausner RD, Sandoval IV. Study of the transit of an integral membrane protein from secretory granules through the plasma membrane of secreting rat basophilic leukemia cells using a specific monoclonal antibody. J Cell Biol 1986;102:516-22.

Chang EH, Masnyk T, Black R, Harford JB. Effects of interferon-gamma on growth of A431 cells and expression of EGF receptors. ICSU Short Reports 1986;4:370-1.

Hentze M, Keim S, Papadopoulos P, O'Brien S, Drysdale D, Leonard WJ, Harford JB, Klausner RD. Cloning, characterization, expression, and chromosomal localization of a human ferritin heavy-chain gene. Proc Natl Acad Sci 1986;83:7226-30.

Klausner RD, Harford JB, Rao K, Mattia E, Weissman AM, Rouault TA, VanRenswoude J, Ashwell G. Molecular aspects of the regulation of cellular iron metabolism. In: Spik G, Montreuil J, Crichton RR, Mazurier J, eds. Proteins of iron metabolism. Amsterdam: Elsevier Science Publishers BV 1985;111-22.

Klausner RD, Dean J. Genetics and metabolic diseases. In: Wyngaarden JB, Smith LH, eds. Cecil review of general internal medicine. 3rd edition. Philadelphia: WB Saunders Co, 1985;145-58.

Leonard WJ. Molecular analysis of the gene encoding the human interleukin-2 receptor. In: Puett D, Ahmad F, Black S, Lopez DM, Melner MH, Scott WA, Whelan WJ, eds. Advances in gene technology: molecular biology of the endocrine system. Proceedings of the eighteenth Miami Winter Symposium. ISCU short reports; vol 4. Boston: Cambridge University Press, 1986;354-357.

Leonard WJ, Depper JM, Kanehisa M, Kronke M, Sullivan M, Peffer N, Svetlik P, Greene W. Structure of the human interleukin-2 receptor gene. Science 1986;230:633-9.

Mattia E, Josic D, Ashwell GG, Klausner RD, van Renswoude J. Regulation of intracellular iron distribution in K562 human erythroleukemia cells. J Biol Chem 1986;261:4587-93.

O'Shea JJ, Harford JB, Klausner RD. Identification and characterization of the phosphatidylinositol kinase in membranes of murine T lymphocytes. J Immunol 1986;137:971-6.

Rao KK, Harford JB, Rouault, T, McClelland A, Ruddle FH, Klausner RD. Transcriptional regulation by iron of the gene for the transferrin receptor. Mol Cell Biol 1986;6:236-40.

Rouault T, Rao K, Harford J, Mattia E, Klausner RD. Hemin, chelatable iron, and the regulation of transferrin receptor biosynthesis. J Biol Chem 1985;260:14862-6.

Samelson LE, Harford JB, Klausner RD. The components of the murine T cell antigen receptor complex. In: Oppenheim JJ, Jacobs DM, eds. Leukocytes and Host Defense. NY: Alan R Liss, Inc, 1986;37-45.

Samelson L, Harford J, Klausner R. Identification of the components of the murine T cell antigen receptor complex. Cell 1985;43:223-31.

Samelson LE, Patel MD, Weissman AM, Harford JB, Klausner RD. Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. Cell 1986;46:1083-90.

Samelson LE, Weissman A, Robey FA, Berkower I, Klausner RD. Characterization of an antibody that recognizes the murine analogue of the human T cell antigen receptor-T3 delta chain. J Immunol 1986;137:3254-8.

Sharon M, Klausner RD, Cullen B, Chizzonite R, Leonard WJ. A novel subunit of the interleukin-2 receptor detected by cross-linking under high affinity conditions. Science 1986;234:859-63.

Weissman AM, Harford JB, Svetlik PB, Leonard WJ, Depper JM, Waldmann TA, Greene WC, Klausner RD. Only high-affinity receptors for interleukin 2 mediate internalization of ligand. Proc Natl Acad Sci 1986;83:1463-6.

Weissman AM, Klausner RD, Rao KK, Harford JB. Exposure of K562 cells to anti-receptor monoclonal antibody OKT9 results in rapid redistribution and enhanced degradation of the transferrin receptor. J Cell Biol 1986;102:951-8.

Weissman AM, Rao KK, Rouault T, Klausner RD, Harford JB. The regulation and dynamics of the transferrin receptor. In: Greten H, Windler E, Beisiegel, eds. Receptor mediated uptake in the liver. Berlin Heidelberg:Springer-Verlag, 1986;154-62.

Laboratory of Developmental and Molecular Immunity--
John Robbins, M.D., Chief

This Laboratory conducts research on the developmental and molecular biology of "natural," disease-acquired and immunization-induced immunity, with especial emphasis on antigens involved in the immunopathogenesis of diseases of the developing organism. Three areas of inquiry have been addressed: (1) Immunopathogenesis of invasive diseases of infants and young children caused by encapsulated bacteria and the development of vaccines for their prevention, and characterization of the mechanisms underlying the age-related development of serum antibody formation to the protective antigens of these pathogens. Methods are being developed to provide more rapid and reliable diagnosis of bacterial infections of infants. The exotoxins of Bordetella pertussis, including pertussis toxin, are studied for their immunopathogenic role in pertussis; (2) The development, activation, timing of expression, regulation of intrauterine and neonatal synthesis, and structure-function relations of the Class I transplantation antigens. Site-directed mutagenesis is used to provide information about the structure-function relations of these polymorphic immunoregulatory molecules, and the effects of interferon, retinoic acid and oncogenes on the synthesis of Class I histocompatibility antigens are being studied by a variety of methods including the novel approach of cloning cells cultured from embryos at crucial periods in the development of these antigens. Critical to this research is the elucidation of the DNA sequences encoding the Class I MHC antigens, specifically those involved in their structure, regulation and intrauterine activation; (3) The immunoregulatory function of bacterial toxins, including pertussis toxin and Group A beta hemolytic streptococcal pyrogenic exotoxin, is being characterized using hybridomas constructed from both T and B lymphoid cells at various stages of their differentiation.

R. Schneerson and J. Robbins continue their work on the pathogenesis and prevention of invasive diseases due to encapsulated bacteria of infants and children. Their emphasis has been primarily on Haemophilus influenzae type b and pneumococcal infections, but has now been extended to Salmonella typhi and related causes of enteric fevers.

The effectiveness of the H. influenzae type b and pneumococcal polysaccharide vaccines in infants and young children is hampered by their age-related relative lack of immunogenicity and the lack of a booster response upon reinjection. Synthetic conjugation schemes were devised by Schneerson and Robbins in order both to increase this immunogenicity in infants and to confer the property of T cell dependence on the capsular polysaccharides of H. influenzae type b and pneumococcus type 6 (as an example of the weakest immunogen and age-dependent component of the pneumococcal vaccine). Following extensive studies of the immunologic and safety properties of these vaccines in laboratory rodents and then primates, a clinical study in normal adults was undertaken. Volunteers were immunized two times with conjugates composed of H. influenzae type b, or the cross-reacting Escherichia coli K100 or pneumococcal type 6A polysaccharides, bound to tetanus toxoid. Local effects were common and probably due to Arthus reactions mediated by high levels of preexisting tetanus toxin antibodies. Fever occurred in about 10% of recipients after the first injection; none was detected after the second injection. The H. influenzae type b-tetanus toxoid conjugate elicited a 180-fold increase in serum polysaccharide antibodies, and the pneumococcus type 6A conjugate elicited an 8-fold increase. Each conjugate elicited about a 10- to 20-fold increase in serum tetanus toxin

antibodies. Similar levels of antibodies to the polysaccharide and tetanus toxoid components were elicited by 50 and 100 microgram doses, and by one conjugate or a combination of conjugates. Pre- and post-immunization sera had H. influenzae type b antibodies composed of IgG, IgA and IgM immunoglobulins. Immunization with either the purified polysaccharide or the conjugates elicited an increase in all three isotypes; the greatest increase was in the IgG component. The highest response in this latter isotype was in the IgG2 subclass. A one way cross-reaction was apparent since about one-half of the volunteers immunized with the pneumococcus type 6A conjugate responded with 2-fold or greater levels of H. influenzae type b antibodies. The polysaccharide and tetanus toxoid antibodies elicited by the conjugates had biological activities that have been correlated with immunity to infection by their respective agents. The conclusion from these first clinical studies is that the quality of the antibodies elicited by the conjugates is similar to that elicited by the polysaccharide component injected alone; the level of polysaccharide antibodies stimulated by the conjugates was considerably higher. Phase 2 studies of these conjugates in children are ongoing. The results to date are encouraging and suggest that it will be possible to devise polysaccharide vaccines that are effective in infants, who remain at serious risk of morbidity and mortality from these infections despite the availability of antibiotics.

Preliminary evaluation of these conjugates in patients with immunodeficiency disorders and recurrent infections has been done in collaboration with Dr. Lars A. Hanson, University of Goteborg (Sweden). Most patients responded with levels of H. influenzae or type 6 antibodies in a manner similar to that of normal volunteers; two patients with non-detectable or very low levels of IgG2 showed a considerably lower response, but still at levels considered to be protective. Another collaborative study, with Drs. Sarnack and Kaplan of Wayne State University, is planned to evaluate these conjugates in infants and children with sickle cell anemia, another disease that predisposes to an unusually high rate of invasive infections by encapsulated bacteria.

As in the case of other encapsulated bacterial pathogens, capsular polysaccharide antibodies confer immunity to Neisseria meningitidis. In contrast to the other serogroups, meningococcal Group A diseases have a unique epidemiology: In Africa, Group A meningitis is endemic with a high frequency. In other parts of the world, Group A causes epidemics. In both situations, asymptomatic carriage is low. In the U.S., Group A meningococci carriers have not been detected for the past 30 years. Most young adults have Group A polysaccharide antibodies. Yet, the antigenic stimuli for these antibodies has not been identified. Two Escherichia coli capsular polysaccharides, K93 and K51, were discovered which could account for this ubiquitous "natural" immunity. Their serological properties and structures were characterized. Schemes for synthesis of K93, K51 and Group A polysaccharide conjugates have been devised in order to provide more effective immunity against Group A meningitis in infants and children than provided by the current Group A vaccine.

As noted previously pneumococcal infections cause considerable morbidity and continuing mortality, especially in infants and young children, despite effective antibiotics and supportive care. The limitations of the pneumococcal vaccine (vide supra) prompted a search for a more immunogenic and species-specific, rather than a multivalent type-specific, immunogen. Briles et al. showed that monoclonal antibodies, specific for phosphocholine (PC), conferred

protection in mice against several pneumococcal types. The most likely candidate for this PC-containing protective antigen is the cell wall polysaccharide (Cw-Ps). The Cw-Ps, present in all pneumococci, is a linear copolymer which contains PC bound to its galactose residue. The Cw-Ps was bound to a carrier protein through the use of a heterobifunctional coupling agent, N-succinimidyl 3-(2-pyridydithio)-proprionate (SPDP). The resultant Cw-Ps protein conjugate was injected into animals and the hyperimmune antiserum assayed for its ability to confer passive protection against lethal challenge with several pneumococcal types in a standardized mouse model. The conjugate elicited Cw-Ps antibodies without PC specificity and did not confer protection. However, CW-Ps antibodies elicited by multiple intravenous injection of a mutant pneumococcus strain, SRC-2, which contains a capsule composed of the Cw-Ps,did elicit antibodies with PC specificity. This antiserum was protective, but absorption with Cw-Ps did not change its protective activity. These data provide evidence that antibodies to the Cw-Ps do not confer immunity against infection of mice with encapsulated pneumococci. A search for the protective activities of other PC-containing structures is ongoing.

Enteric fevers have a similar pathogenesis and clinical picture, and are caused by Salmonellae. In developing nations, the most common (usually accounting for 80% of enteric fevers) and most severe is typhoid fever. Typhoid fever continues to be a problem for U.S. citizens in countries where this infection is epidemic. Transmission of Salmonella typhi, the causative agent of typhoid fever, is almost always by contaminated water. Control of enteric fevers has been achieved where hygenic practices have been applied to food handling, disposal of sewage, and monitoring of patients. However, it is unlikely that control will be achieved in developing nations in the near future, other than by vaccination. Current typhoid vaccines are unsatisfactory because they induce a high rate of side reactions and confer only a limited immunity.

S. typhi is pathogenic for humans only; there are no satisfactory animal models. S. typhi has a capsular polysaccharide, called the Vi (virulence) antigen; no other Salmonellae have a comparable structure. Circumstantial evidence has been amassed to suggest that antibodies to the Vi antigen confer immunity to typhoid fever. Accordingly, methods were devised for the production, isolation, standardization, and evaluation of the effectiveness of the Vi polysaccharide in preventing typhoid fever. The Vi vaccine was standardized by methods established for other polysaccharides and by ^{14}C nuclear magnetic resonance spectroscopy. Clinical safety and immunogenicity trials have been conducted in medical students in Lyon, France, and in school children and adults, ages 5 through 45 years, in various villages of the Kathmandu Valley, Nepal. The results were encouraging, and consequently, the effectiveness of the Vi polyaccharide in preventing typhoid fever has now been evaluated in a clinical study established in five villages, comprising a total population of about 13,000, in Nepal. Seven thousand volunteers received either 25 micrograms of the Vi polysaccharide or a pneumococcal vaccine (control) in a randomized and double-masked fashion. No serious reactions were encountered. Local swelling and redness occurrred in about 10% of the vaccinates,most prominantly in the adults. The reactions were not incapacitating and waned in 48 hours. Their timing and appearance were consistent with the reactivity usually elicited by pneumococcal vaccines in adults. Surveillance will be maintained in the five villages that participated in the effectiveness trial for at least two years. If effectiveness is demonstrated, these workers plan to enlist the

support of the Agency for International Development to maintain this surveillance for an additional five years to ascertain the duration of immunity. As the routine of surveillance becomes established, this group hopes to study the immunological characteristics, and genetic regulation of these characteristics, in patients with typhoid fever.

It is stated that typhoid fever is unusual under the age of five years. This is unexpected because other infectious diseases, contracted by ingestion of contaminated water, occur with high frequency in this age group. This group plans to study the epidemiology of typhoid fever and other causes of enteric fevers in this age group using more reliable methods of diagnosis including bone marrow culture. Based upon these studies and the results of our effectiveness study in Nepal, these workers will judge whether or not the Vi alone could be studied for its effectiveness in preventing typhoid fever in infants and children up to the age of five years. They have developed methods for binding capsular polysaccharides that contain an uronic acid residue (e.g., the Vi polysaccharide) to medically useful carrier proteins in order to make them effective vaccines for infants and young children. The safety, immunogenicity, and the effectiveness of these newly synthesized Vi-protein conjugates could then be considered for evaluation in the very young.

The incidence of pertussis, a communicable disease with high morbidity and mortality in infants and young children, has been controlled by vaccination. The current pertussis vaccine is composed of inactivated Bordetella pertussis organisms and is a component of the triple formulation, DTP, given routinely in the USA to infants and children. The pertussis component, however, suffers from two limitations; (1) It induces a high rate of side reactions, including a rare encephalopathy; and (2) the extent and duration of protection are limited compared to other vaccines. The side reactions have caused increasing public concern and the use and supply of pertussis vaccines is decreasing consequently. Recently, Pittman proposed that a single component of B. pertussis, pertussis toxin (PT), is the major immunopathogenic determinant. Acting on Pittman's proposal that pertussis is a toxin-mediated disease, and that serum PT antibodies will confer immunity, R. Sekura has developed methods for production of PT in quantities and purity suitable for clinical investigation. PT has an enzymatic action, ADP ribosylation, similar to other bacterial exotoxins including those of C. diphtheriae and V. cholera. The specificity of binding of PT to mammalian cells was studied using the serum glycoprotein, fetuin, as a model compound. Binding of PT to fetuin was shown to be due to its interaction with the asparagine-linked oligosaccharide of this glycoprotein. A novel method was devised to inactivate both the binding and enzymatic activities of PT while retaining at least one-third of its antigenicity. The detoxified PT induced active immunity against challenge with B. pertussis by the intracerebral and pulmonary routes in laboratory mice. A lot of PT, PTH-06, prepared and standardized for clinical evaluation, showed no toxicity and induced neutralizing antibodies in primates. Assays to monitor the antigenicity, immunogenicity, binding and enzymatic activity of the inactivated PT (pertussis toxoid, or PTd) were developed and their usefulness and reliability in standardization tests were confirmed with several production lots. Clinical evaluation was started in adult volunteers at the Clinical Center of the NIH. Three doses, ranging from 10 to 75 μg of PTd adsorbed on aluminum salts, were injected into 25 volunteers each. No local or systemic reactions, alteration in glucose metabolism or changes in lymphocyte counts were observed. Serological responses have been extremely encouraging. It is planned to start Phase II

studies in children in Goteborg, Sweden, in the next several months, assuming that the serum antibody responses elicited by the experimental PTd continue to be similar to those observed in mice and primates.

B. pertussis is a pathogen for humans only; clinical pertussis has not been established in other animals. In addition to PT, this pathogen secretes a variety of biologically active extracellular materials, including adenylate cyclase and an activity referred to as dermonecrotic or heat labile toxin (HLT). This latter activity has not been characterized, but Sekura and his associates have now isolated a protein toxin from sonicates of B. pertussis which has this activity. Homogeneous preparations of HLT have been obtained and it was established that the toxin is a polypeptide with a molecular weight of about 150,000. Purified HLT has been used to produce polyclonal antibodies in rabbits and monoclonal antibodies from hybridoma cultures. Passive immunization of mice with polyclonal HLT antibodies conferred protein against challenge with B. pertussis, suggesting that HLT may serve as a protective antigen in addition to PT. Sublethal doses of HLT, injected into mice, produced unexpected results. Within eight hours, a 70% reduction in the weight of the spleen was observed. Most of the loss in spleen weight was due to reduction in the red pulp (erythropoietic tissue). The lethal dose of HLT in mice was about 0.5 µg, similar to the toxicity of tetanus toxin. HLT antibodies conferred protection against the lethal effects of toxin challenge (anti-toxin). Sekura speculates that HLT induces local inflammation at the site of initial infection with B. pertussis. This local inflammatory action may account for the early non-specific respiratory symptoms of infection with B. pertussis. Studies are underway both to measure HLT antibodies in patients with pertussis and to assess their potential for inducing protective immunity.

The section led by K. Ozato continues to investigate the structure-function relations and developmental regulation of the Major Histocompatibility Complex Class I antigens (MHC). These cell surface molecules are membrane glycoproteins critical to tissue rejection and cell-bound antigen recognition. Class I MHC antigens are composed of three external domains and one membrane-associated domain. The notable structural feature of MHC Class I genes is their extraordinary degree of polymorphism. This high level of polymorphism has been maintained throughout evolution of the vertebrates and cannot be explained by simple genetic drift. MHC Class I antigens present cell-bound antigens (as, for example, by macrophages) and by so doing, activate T cells. This activity is mediated by the polymorphic portion of MHC since Class I MHC mis-matched cells fail to stimulate T cells. Variability is found in Class I MHC genes throughout the first and second domains, making it difficult to identify the regions responsible for MHC immunological functions.

Site-directed mutagenesis was used to study the structure-function relations of Class I MHC genes. Mutant H-2L^d genes were prepared in which several amino acids in the most polymorphic portions of the first external domain were replaced by amino acids of other Class I MHC genes. A 1.9 kb Xba fragment of the H-2L^d gene, containing the first and second exons cloned in M13, served as a template. Synthetic nucleotides of 27 base pairs, containing 2 to 3 nucleotide mismatches, were annealed to the templates and the mutants were identified by dot hybridization. The putative mutants were analyzed by DNA sequencing. The mutants, once identified, were introduced into murine L cells by co-infection with the Herpes thymidine kinase gene. Radioimmunobinding, cytofluorography and reactivity with CTL lymphocytes were used to analyze the expression of

these mutant genes. This approach has been very productive: Ozato et al. have found that a small stretch of amino acid sequences is responsible for a number of antigenic determinants recognized by monoclonal antibodies and by CTL. Furthermore, changes in glycosylation sites and disulfide bridges introduced in this system have revealed information about the location of antigenic sites and intracellular transportation of these antigens.

Ozato has found that murine alpha, beta and gamma interferons (IFNs) induce expression of the c-fos oncogene in undifferentiated embryonal teratocarcinoma (F9 EC) cells, NIH 3T3 cells, and a variety of lymphoid cells. IFN-induced expression is dose-dependent, rapid and transient; the amount of c-fos transcript rises within 15 minutes, peaks after 30 minutes, and soon drops to an undetectable level. The c-fos gene reveals a stretch of 10 nucleotides in the 5' flanking region of the gene homologous to a part of the "IFN-consensus sequence." This and other findings indicate that c-fos is a member of the family of IFN-inducible genes, and that its expression is an early event of IFN action. In mice, c-fos mRNA increases steeply in several tissues on the day of birth, followed by a precipitous decline soon thereafter. Immediately following this burst of c-fos expression, the level of MHC Class I RNA, also known to be induced by IFNs, rises sharply in the neonatal organs. These findings suggest that the sequential expression of c-fos and MHC Class I genes is regulated by IFNs, which are an integral component of neonatal development.

Murine MHC Class I genes are expressed only after the embryos reach mid-somite stage. Since there are no established cell lines representative of embryos at the mid-somite stage, a number of clones from mouse embryos at day 8 and day 11 were established by infection and transformation with several oncogenic retroviruses. These clones of embryonic cells are morphologically distinct. Of 35 independent clones, 10 have been characterized further and found to have varying expression of MHC Class I and SSEA-1 antigens and laminin. SSEA-1 is expressed in early embryonic cells and is no longer detectable after differentiation. Laminin has been shown to be undetectable in early embryos; it is expressed in epithelial cells and later embryos. All ten clones were negative for SSEA-1. Laminin was detected in only some of these ten clones. Unlike F9 EC cells, these clones did not show morphological changes after treatment with retinoic acid. These cells, therefore, appear to retain the characteristics of mid-gestational embryos. The majority of these clones have low levels of MHC Class I mRNA, equivalent to F9 EC cells. Several clones negative for surface MHC Class I antigens showed relatively high constitutive expression of Class I MHC mRNA. Interferon tratment of these clones resulted in an increase in the level of MHC Class I antigens. The kinetics of their IFN-induced MHC Class I surface antigens and mRNA were variable. CAT assay of these clones, seeking a negative regulatory factor apparently present in the undifferentiated F9 EC cells) is underway (vide infra).

The development and expression of MHC Class I antigens was also analyzed, using F9 EC cells to study their gene regulation. The promoter activity of MHC Class I genes was analyzed by employing chloramphenicol acetyl transferase (CAT) fusion genes in a transient transfection system. Sequence data was obtained from the upstream region of the H-2L^d gene in order to identify controlling elements. Portions of the upstream region were fused to the coding sequence of the bacterial CAT gene. Another series of CAT constructs was prepared in which

portions of the SV40 promoter were replaced by the MHC Class I promoter sequences. The promoter activity of the above constructs was assessed in F9 EC cells by measuring transient CAT activity after DNA-mediated gene transfer.

The promoter activity of an MHC Class I gene was studied with F9 EC cells, employing CAT assays with hybrid genes that contained upstream regions of an MHC Class I H-2L^d gene. The 1.4 kb 5' flanking region of the H-2L^d gene revealed the presence of 5-10 bp direct or inverted repeats about 100-230 bp upstream of the gene and immediately adjacent to the IFN consensus sequence. Chimeric genes, in which the CAT genes were under the control of various lengths of the 5' flanking region of the H-2L^d gene, were introduced in F9 EC cells and the promoter activity was estimated by measuring transient expression of the CAT gene. Ozato and her colleagues found that the upstream region of the H-2L^d gene has weak promoter activity in F9 EC cells. When the upstream sequence distal from position 135 (from the cap site) was removed, a 4- to 5-fold increase in CAT activity ensued. Studies with internal deletions indicated that the presence of a 75 bp sequence, encompassing nucleotide positions -135 to -210 from the cap site, represses CAT activity in undifferentiated F9 cells. Upstream fragments that lack this region consistently elicited 4- to 5-fold higher CAT activity. This repression was demonstrable in undifferentiated F9 cells only; all of the constructs had similar CAT activity when the F9 cells had been differentiated by treatment with retinoic acid. This finding indicates that the repressed promoter of the MHC Class I gene is developmentally regulated. The region responsible for this repression contains inverted and direct repeats comprising 5-10 bp nucleotide streches, and the IFN consensus sequence in the proximal region exerts enhancer-like activity in differentiated cells only.

Ozato et al. will continue to study the developmental regulation of MHC Class I genes. Her working hypothesis is that the regulatory region in the 5' flanking region of the H-2L^d gene interacts with a transacting nuclear protein(s) present in early embryonic cells. The proto-oncogene, c-fos, is expressed during murine embryonic development. The c-fos gene is also induced by other growth stimuli in many types of cultured cells. Although c-fos has been implicated in differentiation, no precise function of this regulatory gene has been demonstrated. Ozato proposes to introduce the c-fos gene, under the control of the H-2L^d promoter, into fertilized mouse embryos and to produce transgenic mice. Construction of this hybrid gene has been completed, and its expression is being evaluated in F9 EC cells to confirm its predicted developmental role. This construct will be introduced into C57BL/6 embryos which will then be allowed to develop in foster mothers. The information derived from this new model will be evaluated together with the DNA analysis of cells in culture, including the embryonal cell cultures developed by this laboratory.

During the course of systemic infections, lymphoid cell function responsive to the offending agent and other antigens is often altered (deregulated). In many instances, this deregulation may affect the primary course of the disease or predispose to secondary complications. E. Hanna and his associates study how microorganisms or their products alter the regulation of lymphoid cell structure and function. Hybridomas have been constructed from spleen cell cultures which represent precursor and mature regulatory T-cells. The spleen cell cultures, used to supply the functional cell type of the hybridoma, were either stimulated by T-cell growth factors or antigens, and fractionated into homogeneous cell types prior to the fusion. Cell surface architecture was

analyzed by dual laser, computer-controlled cell sorter analysis with fluorochrome-labeled monoclonal antibodies. Lymphoid function was measured by a variety of in vitro and in vivo standardized techniques, including generation and function of cytotoxic T lymphocytes (CTL) and antibody formation. Two bacterial products have been studied in detail; pertussis toxin (PT) and streptococcal pyrogenic exotoxin (SPE). Both toxins exert stimulatory as well as suppressive activities on various immune functions when injected in vivo; these effects are complex and depend upon the dose, timing and type of immune function that is assayed. Both protein toxins are mitogenic at low concentrations and not cytotoxic at doses that induce effects upon lymphoid function. Both toxins are thought to exert a major immunopathogenic role during diseases caused by their parent bacteria. SPE exerts its effect upon precursor T-cells that are down-regulatory for antibody formation. Exposure of one hybridoma, a precursor T-cell with the differentiation markers Thy1, L3T4 and Lyt 2, results in the reduction of its suppressive activity. The SPE-induced change of this precursor T-cell hybridoma results in reduced expression of both its Lyt 2 marker and its suppressor activity for antibody formation by B-lymphocytes. This effect is consistent with the capacity of SPE to divert the development of function in T-cell precursors. The SPE-induced suppression of CTL, in contrast, was not as marked as that mediated by PT. PT exerts its immunomodulating effect by generating an increase in suppressor cell types. Spleen cell cultures, exposed to this toxin, show an increase in $L3T4^{(-)}$, $Lyt2^{+}$, $Thy1^{+}$, T-cells. These cells have been partially purified and found to exert a suppressive effect upon generation of CTL's in cell culture.

Certain stress mimicking compounds, such as N'methyl-B-carboline-3-carboxamide, exert modulatory effects upon lymphoid function. Treatment of animals with this compound suppressed the generation of CTL's. This in vivo effect did not occur when the compound was added directly to spleen cultures capable of generating CTL. The intermediate biological mediator of this suppressive effect remains to be elucidated. Hanna and his colleagues are seeking an understanding of the biological intermediates and will employ their library of precursor and T-cell clones and hybridomas to identify the cellular basis for this immunodulating effect.

Laboratory of Developmental and Molecular Immunity

Chief, *John B. Robbins, M.D.

Senior Staff

*Edgar E. Hanna, Ph.D.
*Keiko Ozato, Ph.D.
Rachel Schneerson, M.D.
Ronald D. Sekura, Ph.D.

Fellows

Prince K. Arora, Ph.D.
Shousun C. Szu, Ph.D.

Visiting Program

Ali I. Fattom, Ph.D.
Ben-Zion Levi, Ph.D.
Jun-ichi Miyazaki, M.D.
Yasuaki Shirayoshi, Ph.D.
Yong Hong Yang, M.D.
Yan-ling Zhang, Ph.D.

Guest Researchers

Peter A. Burke, M.D.
Elaine M. Daniels, M.D.
John W. Kasik, M.D.
Yael Kaufmann, Ph.D.
Allison Knight, Ph.D.
Xiuru Li, Ph.D.
Toby A. Silverman, M.D.
Audrey L. Stone, Ph.D.
Kenji Sugita, Ph.D.

Bibliography

Birnbaumer L, Codina J, Sunyer T, Hildebrandt JD, Sekura RD, Cerione RA, Lefkowitz RJ, Caron MG. Dual coupling mechanisms in the regulation of cAMP formation. In: Gotto AM Jr, O'Malley BW, eds. The role of receptors in biology and medicine. NY: Raven Press, 1986;193-211.

Caspi RR, Roberge FG, McAllister CH, El-Saied M, Kuwabara T, Gery I, Hanna EE, Nussenblatt T. T-cell lines mediating experimental autoimmune uveoretinitis (EAU) in the rat. J Immunol 1986;136:928-33.

Downs RW, Sekura RD, Levine MA, Spiegel AM. The inhibitory adenylate cyclase coupling protein in pseudohypoparathroidism. J Clin Endocrinol Metab 1985;61:351-4.

Guirguis N, Schneerson R, Bax A, Egan W, Robbins JB, Shiloach J, Orskov I, Orskov F, El Kholy A. Escherichia coli K51 and K93 capsular polysaccharides are crossreactive with the group A capsular polysaccharide of Neisseria meningitidis. Immunochemical, biological, and epidemiological studies. J Exp Med 1985;162:1837-51.

Kabat EA, Nickerson KG, Liao J, Grossbard L, Osserman EF, Glickman E, Chess L, Robbins JB, Schneerson R, Yang YH. A human monoclonal macroglobulin with specificity for α(2-8)-linked poly-N-acetyl neuraminic acid,the capsular polysaccharide of group B meningococci and Eschericia coli K1, which cross-reacts with polynucleotides and with denatured DNA. J Exp Med 1986;164: 642-54.

Koeller D, Ozato K. Evaluation of the structure function relationships of MHC class I antigens by molecular genetics techniques. In: Cruse JM, Lewis RE Jr, eds. The year in immunology. Basel: S. Karger, 1986;2:195-204.

Maloy WL, Ozato K, Sachs DH, Coligan JE. Evidence for the association of I-A and I-E molecules in d-haplotype mice. Mol Immunol 1986;23:263-9.

Mattera R, Codina J, Sekura RD, Birnbaumer L. The interaction of nucleotides with pertussis toxin. J Biol Chem 1986;261:11173-9.

McAllister CG, Vistica BP, Sekura RD, Kuwabara T, Gery I. The effects of pertussis toxin on the inoculation and transfer of experimental autoimmune uveoretinitis. Clin Immunol Immunopathol 1986;39:329-36.

Miyazaki J, Appella E, Ozato K. Intracellular transport blockade caused by disruption of the disulfide bridge in the third external domain of MHC class I antigens. Proc Natl Acad Sci 1986;83:757-61.

Miyazaki J, Appella E, Zhao H, Forman J, and Ozato K. Expression and function of a nonglycosylated major histocompatibility class I antigen. J Exp Med 1986;163:856-71.

Miyazaki J-i, Ozato K. Major histocompatibility class I antigens (review). Med Immunol 1985;9:459-66.

Robbins FC, Robbins JB. Current status and prospects for some improved and new bacterial vaccines. Ann Rev Public Health 1986;7:105-25.

Robbins JB, Schneerson R, Sekura R, Trollfors B, Zhang Y-L, Shiloach J. Vaccines for the prevention of diseases due to Haemophilus influenzae type b and other encapsulated bacteria and Bordetella pertussis in infants and children. In: Tyrell DAJ, ed. Clinical investigation of infectious diseases. Royal Society of Medicine Services International Congress and Symposium Series No. 98. London: Royal Society of Medicine Services Ltd, 1986;153-73.

Schneerson R, Robbins JB, Parke JC Jr, Bell C, Schlesselman JJ, Sutton A, Wang Z, Schiffman G, Karpas A, Shiloach J. Quantitative and qualitative analyses of serum antibodies elicited in adults by Haemophilus influenzae type b and pneumococcus type 6A capsular polysaccharide-tetanus toxoid conjugates. Infect Immun 1986;52:519-28.

Sekura RD, Quentin-Millet M-J. Pertussis toxin: identification of the carbohydrate receptor. In: Lark DL, Normark S, Uhlin BE, Wolf-Watz H, eds. Protein-carbohydrate interactions in biological systems. London: Academic Press, 1986;241-2.

Szu SC, Schneerson R, Robbins JB. Rabbit antibodies to the cell wall polysaccharide of Streptococcus pneumoniae fail to protect mice from lethal infection with encapsulated pneumococci. Infect Immun 1986;54:545-53.

Szu SC, Zon G, Schneerson R, Robbins JB. Ultrasonic irradiation of bacterial polysaccharides. Characterization of the depolymerized products and some applications of the process. Carbohydr Res 1986;152:7-20.

Tacket CO, Ferreccio C, Robbins JB, Tsai CM, Schulz D, Cadoz M, Goudeau A, Levine MM. Safety and immunogenicity of two Salmonella typhi Vi capsular polysaccharide vaccines. J Infect Dis 1986;154:342-45.

Developmental Endocrinology Branch--
D. Lynn Loriaux, M.D., Ph.D., Chief

The research goal of this Branch is to further our understanding of the role of the endocrine tissues in the complex processes of growth, development and reproduction. The endocrine system is being studied in fetal and neonatal life, childhood, young adulthood and old age. Much of the current investigation is focused on the pubertal transition. Systems under study include the hypothalamic-pituitary-gonadal axis, the hypothalamic-pituitary-adrenal axis, the hypothalamic-pituitary-thyroid axis, the systems regulating growth, and placental-fetal interactions. Most of this Branch's work includes clinical research and clinical trials; the major projects reflect the participation of all of the Senior Investigators, including L. Loriaux, G. Cutler, R. Sherins, B. Nisula, F. Cassorla, G. Chrousos, R. Knazek, and G. Merriam. Therefore, results are grouped by topic rather than investigator.

Hormone Transport and Action

The disorders and physiologic processes of growth and reproduction can be investigated in terms of the action of the steroid and glycoprotein hormones. In an attempt to increase our understanding of these processes, this group has examined the relationship between hormone structure and function using the glycoprotein hormones and binding globulins as models.

It was found this year that the alpha-subunit derived from intact human chorionic gonadotropin (hCG) contains oligosaccharide side chains that are primarily terminated in a single sialic acid (i.e., monosialylated). This was unexpected since the structure of the oligosaccharides was believed to be bifurcated, both chains ending in a sialic acid residue (i.e., disialylated). Apart from the value of this finding to structural biochemistry, it contributes to a growing body of evidence supporting a role for the carbohydrate of hCG in receptor binding, target cell activation, metabolism, and combination with complimentary subunits. In contrast, most of the free alpha-subunit oligosaccharides (subunits not derived from intact hCG) are di-and trisialylated. Since the more heavily glycosylated free alpha-subunits are incapable of combining with native hCG beta-subunits to form the intact hormone, it is possible that the carbohydrate on the free alpha-subunit serves to regulate net hCG synthesis by preventing combination with the hCG beta-subunit. These studies have opened new areas of investigation into the regulation of hCG biosynthesis.

Previous studies of the thyrotropic activity intrinsic to hCG showed that removal of its carbohydrate converts hCG from an agonist to an antagonist of TSH action. Thus, these workers hypothesized that the carbohydrate moieties of TSH would also play a critical role in the specific activity resulting from the binding of TSH to its receptor. This has proved to be the case. Studies of the functional properties of the deglycosylated TSH were performed using human thyroid membranes. Deglycosylated TSH bound to TSH receptors in thyroid membranes with more than twice the affinity of native TSH. Despite its enhanced binding affinity, however, deglycosylated TSH was able to stimulate only 10% of the activity of the adenylate cyclase activity achieved with native TSH. The deglycosylated TSH also exhibited antagonistic activity toward TSH-stimulated adenylate cyclase activity. These findings provide the first direct evidence for the role of the TSH carbohydrate moieties in the transduction of the TSH

hormonal signal across the thyroid cell membrane.' From the clinical perspective, these data provide a rational basis for the development of a TSH antagonist that could be used in the treatment of hyperthyroidism.

In healthy human subjects, TSH is secreted in a circadian pattern. The lowest level occurs at about 5:00 in the afternoon and the peak occurs between midnight and 4:00 in the morning. The progressive rise in the serum TSH concentration from 5:00 to midnight is called the "nocturnal TSH surge." Thyroid gland function exhibits a similar pattern. The nocturnal TSH surge is presumably related to a signal generator in the central nervous system, like many other biologic rhythms. Awareness of this phenomenon prompted these investigators to predict that certain diseases of the central nervous system might interfere with the signals that mediate the nocturnal surge and thus lead to hypothyroidism. The nocturnal surge of TSH was examined in a series of 16 patients with hypothalamic and/or pituitary disease. Six of the patients were hypothyroid. The nocturnal TSH surge was absent in the 6 hypothyroid patients and present in 10 euthyroid patients. The significance of this finding derives from its clarification of the mechanism of hypothyroidism associated with hypothalamic disease, and its potential use as a clinical test for patients with hypothalamic and/or pituitary disease who are at risk of developing central hypothyroidism.

The total amount of hormone in plasma often does not reflect its bioavailability. This is because only the "free hormone" (unbound hormone) appears to be available to the tissues. Accurate measurement of the free hormone has been impeded by the lack of availability of ultrapure radioligands. The impurities, often only a small fraction of the total radioactivity, produce large errors in the measurement of free steroid hormone. These workers have now devised a practical procedure to separate radioactive impurities from steroid radioligands. The technique employs a solid phase Sepharose-Con A-binding globulin to precipitate the steroid of interest; non-binding species are excluded, and the binding species of interest is purified. This improved assay system has been used to study the binding globulins in a number of disorders. The levels of most binding globulins are regulated by specific physiologic, ontologic and endocrinologic factors. For example, a rapid dose-related response of sex hormone binding globulin (SHBG) to treatment with thyroxine was demonstrated in familial dysalbuminemic hyperthyroxinemia. This was the first demonstration of thyroid hormone responsivity in patients with this condition. Evidence that the adrenal axis modulates SHBG and cortisol binding globulin (CBG) levels has also been obtained. The levels of both of these steroid binding proteins increase in patients with Cushing's disease during blockade of glucocorticoid action with the receptor antagonist RU 486. This observation suggests that levels of these proteins in plasma may serve as measures of response to therapy, and provide new tools to assess the adequacy of several commonly used treatment regimens for glucocorticoid excess.

Studies on Growth

The primary focus of the current studies on growth is to understand the relative roles of the steroid hormones and the various growth factors in regulating skeletal growth and epiphysial maturation. One of the critical problems in the study of growth is that the process is slow. This is reflected in the difficulty of making reliable measurements over short periods of time. These workers have sought to solve this problem by using new devices that measure the

length of a single bone or limb segment with great precision. To examine the utility of these assays, stature and lower leg length were measured in 13 normal prepubertal children at three week intervals for one year. Height velocity at three week intervals did not show significant differences across the year. At six and twelve week intervals, spring and autumn growth velocities were higher; however, the amplitude of the variations was small. These data suggest that, contrary to earlier belief, only minimal variations in growth velocity occur during the year. There is no evidence of a major seasonal influence on growth. Using this methodology, this group has begun to study the mechanism of pubertal growth. The primary objective is to define the relative roles of the steroid sex hormones, growth hormone, and the insulin-like growth factors (IGF-1, IGF-II).

To evaluate the hypothesis that sex steroids increase spontaneous growth hormone secretion, GH secretion and GH responses to standard provocative tests were measured in twenty-one children with idiopathic short stature. Eight of these children were pretreated with sex hormone (estrogens for girls, androgens for boys). All children were prepubertal and had no endocrine abnormalities. The children who received sex steroid pretreatment showed a greater frequency of growth hormone secretory bursts [6.0 ± 0.3 (SEM) vs. 3.2 ± 0.3, $p < 0.02$]. The peak responses of growth hormone to arginine and to L-dopa were higher in sex hormone-treated children (27.3 ± 5.5 ng/ml vs. 9.8 ± 1.9, $p < 0.02$, and 11.4 ± 3.3 vs. 5.7 ± 2.2, respectively). Responses to insulin and to GHRH, however, were not different between the two groups. This data supports the concept that sex steroids stimulate growth hormone secretion, and is compatible with the idea that sex steroids influence growth, at least in part, through altering secretion of growth hormone. The dose-response relationship between sex hormone and growth was examined in children with Turner's syndrome. Previous studies showed that the dose of ethinyl estradiol yielding optimum growth in girls with Turner's syndrome is 10 ng/kg/d. This is considerably lower than the dose currently recommended by most textbooks. These investigators have now examined the effects of low-dose estrogen treatment over a 6-month period. Growth was sustained without accelerating bone age advancement. This result suggests that the ultimate stature in patients with Turner's syndrome may be improved with low-dose estrogen treatment. These data also imply that the currently recommended regimen may be harmful and actually reduce ultimate height, and the findings represent an important advance in the treatment of Turner's syndrome, a disorder characterized by ultimately short stature.

The role of sex steroids in pubertal growth has also been examined by reducing sex steroid secretion in children with precocious puberty through the use of a long acting analogue of LHRH. The results of 2 to 4 years of such therapy have been examined in 27 children with central precocious puberty. Linear growth rates decreased from 11.0 ± 0.8 cm/yr before treatment to 5.7 ± 0.4 cm/yr after two years of treatment ($p < 0.025$). Predicted heights by the Bayley-Pinneau method increased from 156.4 ± 2.0 cm before treatment to 162.3 ± 2.3 cm at two years ($p < 0.001$). Five patients treated for 4 years had a mean increase in predicted height of 5.5 cm. No adverse effects have been observed. This study demonstrates the sustained effectiveness of D-Trp^6-Pro^9-NEt-LHRH ($LHRH_\alpha$) in central precocious puberty over a period of 2 to 4 years, with growth rate and bone maturation slowing and predicted height increasing by 5-7 cm over the first 2-4 years. $LHRH_\alpha$ thus appears to constitute an effective long term therapy for precocious puberty, and its effects on growth are mediated by changes in sex steroid hormones.

The role of growth hormone itself has been explored by altering its concentration with growth hormone releasing hormone (GHRH). An additional goal of these studies has been to determine whether or not GHRH will be an effective therapeutic agent in growth hormone deficiency (GHD). GHRH has previously been shown to accelerate growth in children. GHRH administration by infusion pump or by intravenous injection, however, is demanding and labor-intensive. Subcutaneous administration would be simpler, but the relative efficacy of this approach is unknown. This issue was examined by treating 7 prepubertal GHD children with GHRH, given subcutaneously by pulsatile infusion pumps at 3 µg/kg/pulse or with intermittent subcutaneous injections three or four times a day. Subjects underwent several successive 6-week treatment periods separated by 3-week rest intervals. The pumps delivered up to 16 pulses/day, and were used only at night. Doses ranged from 1 µg/kg/day to 27 µg/kg/day. Height and lower leg measurements were made at 3 week intervals. Both treatments stimulated GH secretion, and both accelerated lower leg growth velocities. Thus, it is not necessary to normalize the daily pattern of growth hormone secretion to stimulate growth, and a once-daily subcutaneous injection regimen of GHRH can be an effective treatment for children with growth hormone deficiency.

Studies on the Disorders of Puberty

One of this Branch's primary research objectives is to understand the physiologic processes underlying the initiation of puberty. As part of this effort, these investigators have examined several clinical disorders of puberty. The McCune-Albright syndrome, for example, comprises a triad of precocious puberty, polyostotic fibrous dysplasia and cafe au lait pigmentation of the skin. The gonadal activity of these patients appears to be independent of pituitary gonadotropin secretion, and this finding led to the hypothesis that some as yet unidentified circulating factor is responsible for the gonadal activation in this syndrome.

To test this hypothesis, sera from patients and matched control subjects were tested in a rat Sertoli cell FSH bioassay. (The assay measures FSH dependent aromatase activity.) McCune-Albright patients had slightly increased FSH bioactivity compared to prepubertal controls, but the level was too low to explain the degree of estradiol elevation seen in this disorder. Patients with familial male precocious puberty were examined in an analogous way, except that LH activity was sought. Sera from 7 patients and from 6 prepubertal control children, matched for gonadotropin level by RIA, were studied. Testosterone secretion exceeded the upper limit for the control samples in four patients. Thus, testis activation in this disorder may be caused by a circulating factor that is not detected by gonadotropin radioimmunoassay. These studies provide new insight into the mechanisms of gonadotropin-independent precocious puberty, and offer a foundation upon which rational therapy can be based. For example, patients with the McCune-Albright syndrome have now been treated successfully with testolactone, an aromatase inhibitor, and patients with familial male precocious puberty have been treated with a combination of testolactone and an antiandrogen. The antiandrogen (spironolactone) decreased androgenic manifestations of precocious puberty, such as acne and spontaneous erections, but did not control accelerated growth and bone maturation. However, testolactone did normalize growth rate and the rate of bone maturation.

Studies of Adrenal Function

The adrenal gland plays an important role in growth, development, and homeostasis. It is regulated by pituitary ACTH and, in turn, hypothalamic CRF. These three elements of adrenal physiology have been studied in several situations associated with altered adrenal function. Cushing's disease is caused by an ACTH-secreting pituitary adenoma. Unless accurately diagnosed and treated, it is lethal, but our ability to diagnose and treat this disorder has now been very much improved. It was found that exogenously administered CRF elevates plasma ACTH and cortisol in patients with Cushing's disease. CRF does not do this in patients with ectopic ACTH secretion. The diagnostic accuracy of the CRF test in this situation exceeds 85 percent. Patients with Cushing's syndrome of adrenal etiology have low levels of plasma ACTH that also fail to respond to exogenous CRF. Thus, the CRF stimulation test is a useful diagnostic tool in the differential diagnosis of Cushing's syndrome, distinguishing pituitary from adrenal or ectopic causes. This is an important result because each lesion requires a different therapeutic approach. A prospective study of this patient series suggests that the CRF test is equal or superior in its predictive value to the standard tests, which are long, cumbersome, and require hospitalization.

Cushing's disease is best treated by transsphenoidal adenomectomy. The cure rate in most centers is about 50%, but this has now been improved to almost 100% by introducing a new sampling method used in association with CRF administration. The veins draining the pituitary gland (petrosal sinuses) are sampled and ACTH is measured simultaneously on both sides before and after CRF. In the majority of patients, an ACTH concentration gradient is found on the side of the adenoma. Thus, the surgeon searches for the adenoma on the side of the gradient and, if he is unable to find it, performs a hemipituitectomy on that side. This new approach has increased the rate of surgical success to just slightly less than 100%.

Patients with psychiatric disorders associated with hypercortisolism (depression, panic anxiety, and anorexia nervosa) have decreased plasma ACTH responses to exogenous CRF. The blunting of the response is inversely proportional to the degree of hypercortisolism. Thus, it may be possible to differentiate patients with a behavioral disorder (i.e., pseudo-Cushing's syndrome) from patients with true Cushing's disease. These results suggest that the corticotroph cell in depression and anorexia nervosa is stimulated by excess endogenous CRF. This has been tested experimentally by administering a continuous infusion of CRF to normal subjects. Mild hypercortisolism, similar in magnitude and secretory pattern to that seen in depression, was the result. Administration of a bolus of CRF at the end of the continuous infusion induced a blunted ACTH and cortisol response, similar to that observed in depression and anorexia nervosa. In this regard, the intracerebroventricular administration of CRF to experimental animals causes behavioral changes which include aggression, irritability, anorexia, and impotence. These are the same manifestations observed in stressed humans. The findings suggest that CRF may play a role in generating the symptoms associated with conditions characterized by chronic hypercortisolism. If this hypothesis is correct, treatment directed toward preventing endogenous CRF hypersecretion may prove beneficial in these conditions. Recent studies suggest that not all CRF is hypothalamic in origin, and the possibility was examined that extra-hypothalamic CRF may play a role in

homeostasis during stress. The known extra-hypothalamic corticotropin-releasing factors appear to have a different immunoreactivity pattern than hypothalamic CRF. Immune system products such as interleukin I and thymosin, or tissue growth factors such as epidermal growth factor, are potential candidates. To this end, these workers demonstrated that epidermal growth factor stimulates ACTH secretion in primate pituitary cells in vitro at doses or concentrations equimolar to those of CRF. It remains to be shown if this phenomenon has physiologic relevance.

In the search for tissue CRF(s), two models of stress were explored. In a surgical model, patients undergoing neck exploration were examined before, during and after surgery. Surprisingly, only mild activation of the HPA axis and the adrenomedullary system were seen during surgery. ACTH and cortisol secretion was continuous rather than pulsatile, the bulk of ACTH and epinephrine secretion taking place during anesthesia reversal.

The second model is exercise. Exercise is associated with the "fight or flight" response, which is known to be activated in an intensity-dependent fashion that correlates well with both the percentage of maximal oxygen consumption and plasma lactate elevation. A treadmill was used to quantitate exercise. Trained subjects could perform with minimal activation of the axis, seemingly the result of chronic adaptation to exercise stress. This was not true for sedentary subjects. There was, in all subjects, a direct relationship between exercise intensity and activities of the HPA axis. Lactate appears to be a good candidate for the messenger molecule. Lactate stimulates pituitary ACTH secretion in vitro and pyruvate does not. Studies that test the action of L-lactate as a CRF in vitro are now in progress. More generally, these results show that exercise and stress induce parallel responses, and that the stress response can be controlled by training.

Studies on Reproduction

Studies on reproduction during the last year have concentrated on the infertile male, and attention has been focused on factors that could explain idiopathic infertility. These investigators found that the motility of sperm from infertile men is the same as that from normal matched controls, suggesting that sperm motion is not an important variable. However, other factors do seem to differentiate the infertile population from normal. These factors include the distribution of epidydimal proteins over the sperm head and the response of the sperm to hyposmotic shock. The biochemical abnormalities underlying these phenomena are unknown, but clarification of these issues holds promise for rational therapy.

The treatment of hypogonadotropic hypogonadism has been traditionally based on replacement therapy with hCG and FSH. Recent studies in other centers have suggested that LHRH given in a pulsatile fashion from an external pump may be superior to the standard regimen of gonadotropins. This group explored this concept using a dose-response study of the effect of LHRH on testis size and sperm count. The results show that testis size is improved by the pump compared to the standard regimen, but that the sperm counts are not different. Since pump treatment is exceedingly labor-intensive, it appears that the traditional treatment may be preferable.

Developmental Endocrinology Branch

Chief, *D. Lynn Loriaux, M.D., Ph.D.

Senior Staff

Fernando Cassorla, M.D.
George P. Chrousos, M.D.
*Gordon B. Cutler, M.D.
Richard A. Knazek, M.D.
*Bruce C. Nisula, M.D.
*Richard J. Sherins, M.D.

Fellows

Jeffry Baron, M.D.
Diana Blithe, Ph.D.
Allen Burris, M.D.
Linda Golden, M.D.
Louisa Laue, M.D.
Barbara Linder, M.D.
Penelope Manasco, M.D
Howard Tracer, M.D.
Randall Whitcomb, M.D.

Visiting Program

Aldo Calogero, M.D.
En-hui Hao, M.D.
Michael Grino, M.D.
Ryoji Hiramatsu, M.D.
Chris Liapi, M.D., Ph.D.
Fathia Mahmoud, M.D.
Saul Malozowski, M.D.
Mercedes Uriarte, M.D.

Expert

George R. Merriam, M.D.
Robert E. Wehmann, M.D., Ph.D.

Guest Researchers

Barry Alberston, Ph.D.
Renato Bernardini, M.D.
Jorge Blaquier, Ph.D.
Henry Bohler, M.D.
Medardo Burgeno, M.D.
C. Sue Carter-Porges, Ph.D.
John Cassels, M.D.
Constance Chik, M.D.
Richard V. Clark, Ph.D.
Hogan Coughran, M.D.
Audrey Cristiano, M.D.

George Daniel, M.D.
Julian Davidson, Ph.D.
Penelope Feuillan, M.D.
Anne Fjellestad-Paulsen, M.D.
Marie Gelato, M.D., Ph.D.
Gail Kalin, Ph.D.
Konstantine Kalogeras, M.D.
Shinichi Kawai, M.D.
Catherine Kinet, M.D.
George Koukoulis, M.D.
Takeo Kunbayashi, M.D.
Mark Lavinge, M.D.
Linda Liu, M.D.
Therese Loughlin, M.D.
Andrew Margoiris, M.D.,Ph.D.
Gabriela Marin de Panza, M.D.
Lynnette K. Nieman, M.D.
Maria P. Platia, M.D.
Douglas Rabin, M.D.
Sally Radovick, M.D.
Song G. Ren, M.D.
Susan R. Rose, M.D.
Judith A. Ross Levine, M.D.
Jon Stolk, M.D., Ph.D.
Jorge Tezon, Ph.D.
Paul D. Tippet, M.D.
David Vantman, M.D.
Donna Vogel, M.D., Ph.D.
Yanwan Wu, M.D.
Yinzhi Zhu, M.D.
Michael Zinaman, M.D.

Bibliography

Amr S, Menenez-Ferreira M, Shimohigashi Y, Chen HC, Nisula CB, Weintraub BD. Activities of deglycosylated thyrotropin at the thyroid membrane receptor-adenylate cyclase system. J Endocrinol Invest 1985;8:537-41.

Avgerinos PC, Schuermeyer TH, Gold PW, Tomai TP, Loriaux DL, Sherins RJ, Cutler GB, Chrousos GP. Pulsatile administration of human corticotropin releasing hormone (hCRH) in patients with secondary adrenal insufficiency: restoration of the normal cortisol secretory pattern. J Clin Endocrinol Metab 1986;62:816-21.

Blithe DL, Caron PJ, Louvet JP, Nisula B. Inhibition of follicle-stimulating hormone/diethylstilbestrol-stimulated ovarian growth by extracts of pregnancy urine. Endocrinology 1986;119:2270-8.

Caron PJ, Nieman LK, Rose SR, Nisula BC. Deficient nocturnal surge of thyrotropin in central hypothyroidism. J Clin Endocrinol Metab 1986;62:960-4.

Caron PJ, Sopko G, Stolk JM, Jacobs DR, Nisula BC. Effect of physical conditioning on measures of thyroid hormone action. Horm Metab Res 1986;18:206-9.

Caruso-Nicoletti M, Hench K, and Cassorla F. Reported vs actual heights in parents of children with short stature. Clin Pediatr 1986;25:54.

Chrousos GP, Calabrese JR, Avgerinos P, Kling MA, Rubinow D, Oldfield EH, Schuermeyer T, Kellner CH, Cutler GB, Loriaux DL, Gold PW. Corticotropin-releasing factor: basic studies and clinical applications. Prog Neuropsychopharmacol Biol Psychiatry 1985;9:349-59.

Chrousos GP, Evans MI, Loriaux DL, McCluskey J, Fletcher JC, Schulman JD. Prenatal therapy in congenital adrenal hyperplasia; attempted prevention of abnormal external genital masculinization by pharmacologic suppression of the fetal adrenal gland in utero. Ann NY Acad Sci 1985;458:156-64.

Chrousos GP, Loriaux DL. Cushing's syndrome. In: Radel RE, ed. Conn's current therapy 1986. Philadelphia: W.B. Saunders Co., 1985;499-502.

Chrousos GP, Loriaux DL, Lipsett MB, eds. Steroid hormone resistance: mechanisms and clinical aspects. NY: Plenum Press, 1986;439.

Chrousos GP, Loriaux DL, Tomita M, Brandon D, Lipsett MB. The new world primates as animal models of cortisol resistance. In: Chrousos GP, Loriaux DL, Lipsett MB, eds. Steroid hormone resistance: mechanisms and clinical aspects. NY: Plenum Press, 1986;129-44.

Chrousos GP, Maclusky N, Brandon D, Tomita M, Loriaux DL, Lipsett MB. Progesterone resistance. In: Chrousos GP, Loriaux DL, Lipsett MB, eds. Steroid hormone resistance: mechanisms and clinical aspects. NY: Plenum Press, 1986;317-28.

Clark RV, Sherins RJ. Use of semen analysis in the evaluation of the infertile couple. In: Santen RJ, Swerdloff RS, eds. Male sexual dysfunction: diagnosis and management of hypogonadism, infertility and impotence. NY: Marcell Dekker, Inc., 1985;253-66.

Comite F, Cassorla F, Barnes KM, Hench KD, Dwyer A, Skerda MK, Loriaux DL, Cutler GB Jr, Pescovitz OH. Luteinizing hormone-releasing hormone analogue therapy of central precocious puberty: long term effect on somatic growth, bone maturation and predicted height. JAMA 1986;255:2613-6.

Cutler GB Jr, Cassorla FG, Levine-Ross J, Pescovitz OH, Barnes KM, Comite F, Feuillan PP, Laue L, Foster CM, Kenigsberg D, Caruso-Nicolette M, Garcia HB, Uriarte M, Hench KD, Skerda MC, Long LM, Loriaux DL. Pubertal growth: physiology and pathophysiology. Recent Prog Horm Res 1986;42:443-70.

Cutler GB Jr, Winterer J, Chrousos GP, Feuillan PP, Loriaux DL. Treatment of adrenal hyperplasia. [Letter to the Editor]. J Pediatr 1986;109:389-90.

D'Agata R, Cassorla F, Mericq V, Munabi AK, Gelato MC, Loriaux DL. Direct effect of the luteinizing hormone releasing hormone analog D-Trp^{6}-Pro^{9}-Net-LHRH on rat testicular steroidogenesis. Steroids 1984;43:201-7.

de Lamirande E, Sherins RJ, Gagnon C. The presence of a motility inhibitor within spermatozoa may explain the poor sperm motility of some infertile men. J Androl 1986;7:215-9.

Eil C, Gamblin GT, Hodge JW, Clark RV, Sherins RJ. Whole cell and nuclear androgen uptake in skin fibroblasts from infertile men. J Androl 1985;6:365-71.

Feuillan PP, Foster CM, Pescovitz OH, Hench KD, Shawker TH, Loriaux DL, Cutler GB Jr. Treatment of precocious puberty in McCune-Albright syndrome with the aromatase inhibitor testolactone. N Engl J Med 1986;315:1115-9.

Feuillan PP, Poth M, Reilly W, Bright G, Loriaux DL, Chrousos GP. Ketoconazole treatment of type 1 autoimmune polyglansular syndrome: effects on pituitary-adrenal axis. J Pediatr 1986;109:363-6.

Foster CM, Feuillan P, Padmanabhan V, Pescovitz OH, Beitins IZ, Comite F, Shawker TH, Loriaux DL, Cutler GB Jr. Ovarian function in girls with McCune-Albright syndrome. Pediatr Res 1986;20:859-63.

Gagnon C, de Lamirande E, Sherins RJ. Positive correlation between the level of protein-carboxyl methylase in spermatozoa and sperm motility. Fertil Steril 1986;45:847-53.

Gelato MC, Malozowski S, Nicoletti MC, Ross Levine J, Pescovitz OH, Rose S, Loriaux DL, Cassorla F, Merriam GR. Growth hormone responses to growth hormone-releasing hormone during pubertal development in normal boys and girls: comparison to idiopathic short stature and GH deficiency. J Clin Endocrinol Metab 1986;63:174-9.

Gelato MC, Merriam GR. Growth hormone-releasing hormone. Annu Rev Physiol 1986;48:569-91.

Hiramatsu R, Nisula BC. Preparation of steroid radioligands for ultrafiltration assays by a solid-phase transport globulin method using concanavalin A-sepharose. Steroids 1986;45:73-89.

Laue L, Comite F, Hench K, Loriaux DL, Cutler Jr GB, Pescovitz OH. Precocious puberty associated with neurofibromatosis and optic gliomas: clinical features and response to long-acting luteinizing hormone releasing hormone analog. Am J Dis Child 1985;139:1097-1100.

Lefort GP, Stolk JM, Nisula BC. Renal metabolism of the beta-subunit of human choriogonadotropin in the rat. Endocrinology 1986;119:924-31.

Levine-Ross J, Schulte HM, Gallucci WT, Cutler GB Jr, Loriaux DL, Chrousos GP. Ovine corticotropin-releasing hormone stimulation test in normal children. J Clin Endocrinol Metab 1986;62:390-2.

Lipsett MB, Chrousos GP, Loriaux DL. Steroid binding proteins in the glucocorticoid resistance syndrome and new world primates. In: O'Malley B, Gotto A, eds. The role of receptors in biology and medicine. NY: Raven press, 1986;51-61.

Lipsett MB, Tomita M, Brandon D, Loriaux DL, Chrousos GP. Cortisol resistance in man. In: Chrousos GP, Loriaux DL, Lipsett MB, eds. Steroid hormone resistance: mechanisms and clinical aspects. NY: Plenum Press, 1986;97-110.

Liu L, Booth JD, Barnes KM, Merriam GR, Gray W, Sherins RJ, Loriaux DL, Cutler GB Jr. Evidence that the synthetic 31-amino acid inhibin-like peptide lacks inhibin activity. Endocrin Res 1985;11:191-7.

Loriaux DL, Cutler GB Jr. Diseases of the adrenal glands. In: Kohler PO, ed. Clinical endocrinology. NY: John Wiley and Sons, Inc., 1986;167-238.

Malozowski S, Mericq V, Cassorla F. Effects of ketoconazole on rat ovarian steroidogenic enzymatic activities. Res Commun Chem Pathol Pharmacol 1986;52:403-5.

Malozowski S, Young I, Garcia H, Simoni C, Loriaux DL, Cassorla F. Effects of ketoconazole on rat testicular steroidogenic enzymatic. Steroids 1985;46:659-63.

Munabi AK, Cassorla F, D'Agata R, Albertson BD, Loriaux DL, Lipsett MB. The effects of temperature on the activity of testicular steroidogenic enzymes. J Androl 1984;43:325-331.

Munabi AK, Feuillan PP, Staton RC, Rodbard D, Chrousos GP, Anderson RE, Strober MD, Loriaux DL, Cutler GB Jr. Adrenal steroid responses to continuous intravenous adrenocorticotropin infusion compared to bolus injection in normal volunteers. J Clin Endocrinol Metab 1986;63:1036-40.

Nagelberg SB, Laue L, Loriaux DL, Liu L, Sherins RJ. Cerebrovascular accident associated with testosterone therapy in a 21-year old hypogonadal man. N Engl J Med 1986;314:649.

Nieman LK, Chrousos GP, Kellner C, Spitz I, Nisula BC, Cutler GB Jr, Merriam GR, Bardin CW, Loriaux DL. Use of the glucocorticoid antagonist RU 486 in the treatment of Cushing's syndrome. In: Baulieu E-E, Segal SJ, eds. The antiprogestin steroid RU 486 and human fertility control. NY: Plenum Publ, 1985;339-45.

Nieman LK, Healy DL, Spitz IM, Merriam GR, Bardin CW, Loriaux DL, Chrousos GP. Use of single doses of the antiprogesterone steroid RU 486 for induction of menstruation in normal women. In: Baulieu E-E, Segal SJ, eds. The antiprogestin steroid RU 486 and human fertility control. NY: Plenum Publ, 1985;279-83.

Pescovitz OH, Cassorla FG, Comite F, Loriaux DL, Cutler GB Jr. LHRH analog treatment of central precocious puberty complicating congenital adrenal hyperplasia. Ann NY Acad Sci 1985;458:174-81.

Pescovitz OH, Comite F, Hench K, Barnes K, McNemar A, Foster C, Kenigsberg D, Loriaux DL, Cutler GB Jr. Review of the NIH experience in precocious puberty: diagnostic subgroups and their response to short-term LHRH analogue therapy. J Pediatr 1986;108:47-54.

Pescovitz OH, Cutler GB Jr, Loriaux DL. Management of precocious puberty. J Pediatr Endocrinol 1985;1:85-94.

Premachandra BN, Wortsman J, Nisula BC, Williams IK. Modulation of thyroid parameters in familial dysalbuminemic hyperthyroxinemia. Metabolism 1986;35:283-8.

Rittmaster RS, Loriaux DL, Cutler GB Jr. Sensitivity of cortisol and adrenal androgens to dexamethasone suppression in hirsute women. J Clin Endocrinol Metab 1985;61:462-6.

Rose SR, Chrousos GP, Cornblath M, Sidbury J. Oral starch in the management of post-operative nesidioblastosis. J Pediatrics 1986;108:97-100.

Rose SR, Randolph J, Schulte HM, White B, Hill S, Eil C, Chandra R, Sonis W, Lee BC, Bercu B, Loriaux DL, Chrousos GP. Testicular dysgenesis and mixed gonadal dysgenesis: biochemical characterization and management of a typical case. Am J Dis Child 1985;1:123-8.

Rosner W, Pugeat MM, Chrousos GP, Khan MS. Steroid binding proteins in primate plasma. Endocrinology 1986;118:513-7.

Ross JL, Schulte HM, Gallucci WT, Cutler GB Jr, Loriaux DL, Chrousos GP. Ovine corticotropin-releasing hormone (CRH) stimulation test in normal children. J Clin Endocrinol Metab 1986;62:390-2.

Schiebinger RJ, Chrousos GP, Cutler GB Jr, Loriaux DL. The effect of serum prolactin on plasma adrenal androgens and the production of metabolic clearance rate of dehydroepiandrosterone sulfate in normal and hyperprolactinemic subjects. J Clin Endocrinol Metab 1986;62:202-9.

Schulte HM, Chrousos GP, Gold PW, Booth JD, Oldfield EH, Cutler GB Jr, Loriaux DL. Continuous administration of synthetic ovine corticotropin-releasing factor in man. J Clin Invest 1985;75:1781-5.

Shawker TH, Garra BS, Loriaux DL, Cutler GB Jr, Ross JL. Ultrasonography of Turner's syndrome. J Utrasound Med 1986;5:125-9.

Sherins RJ, Brown BL. Anatomy, physiology and disorders of the male reproductive system.In: Brown BL, Kerney JJ, eds. Proceedings of a Forensic Science Symposium on the Analysis of Sexual Assualt Evidence. Washington D.C.: Government Printing Office, 1986:21-43.

Shi YF, Patterson AP, Sherins RJ. Increased plasma and pituitary prolactin concentrations in adult male rats with selective elevation of FSH levels may be explained by reduced testosterone and increased estradiol production. J Androl 1986;7:105-11.

Tamarkin L, Baird CJ, Almeida OFX. Melatonin: a coordinating signal for mammalian reproduction. Science 1985;227:714-20.

Thorner MO, Evans WS, Vance ML, Blizzard RM, Rogol AD, Kaiser DL, Johanson A, Frohman LA, Thominet JL, Furlanetto R, Gelato MC, Merriam GR, Goldman JH, Reichlin S, Rivier J, Vale M. Growth hormone releasing factor. In: Raiti S, Tolman R, eds. Proceedings of the III Growth Hormone Conference. NY: Plenum Press, 1986:361-72.

Tomita M, Brandon DD, Chrousos GP, Vingerhoeds CM, Foster C, Fowler D, Loriaux DL, Lipsett MB. Characterization of glucocorticoid receptors in Epstein-Barr virus-tranformed lymphocytes from patients with glucocorticoid resistance and a

glucocorticoid-resistant new world primate species. J Clin Endocrinol Metab 1986;62:1145-54.

Tomita M, Chrousos GP, Brandon DD, Ben-Or S, Foster CM, DeVougn L, Taylor S, Loriaux DL, Lipsett MP. Clucocorticoid receptors in Epstein-Barr virus-transformed human lymphocytes. Horm Metab Res 1985;17:674-8.

Udelsman R, Gallucci WT, Bacher J, Loriaux DL, Chrousos GP. Hemodynamic effects of corticotropin releasing hormone in the anesthetized cynomolgus monkey. Peptides 1986;7:465-71.

Udelsman R, Ram J, Gallucci WT, Gordon A. Lipfort E, Norton JA, Loriaux DL, Chrousos GP. Adaption during surgical stress, a reevaluation of the role of glucocorticoids. J Clin Invest 1986;77:1377-81.

Veldhuis JD, Evans WS, Rogol AD, Drake CR, Thorner MO, Merriam GR, Johnson ML. Am J Physiol (Special Communications) 1984;E554-63.

Vogel DL, Magner JA, Sherins RJ, Weintraub BD. Biosynthesis, glycosylation and secretion of rat luteinizing hormone alpha and beta subunits: differential effects of orchiectomy and gonadotropin-releasing hormone. Endocrinology 1986;119:202-13.

Winterer J, Chrousos GP, Loriaux DL, Cutler GB. The adrenal suppressive effect of hydrocortisone in congenital adrenal hyperplasia. Ann NY Acad Sci 1986;458:182-92.

Winterer J, Gwirtsman HE, George DT, Kaye WH, Loriaux DL, Cutler GB Jr. Adrenocorticotropin-stimulated adrenal androgen secretion in anorexia nervosa: impaired secretion at low weight and normalization after long-term weight recovery. J Clin Endocrinol Metab 1985;61:693-7.

Young I, Malozowski S, Winterer J, Nicoletti M, Caron P, Cassorla F. Effect of malnutrition of rat ovarian steroidogenesis. Nutr Res 1986;6:571-6.

Endocrinology and Reproduction Research Branch--
Kevin Catt, M.D., Ph.D., Chief

The research projects of the Endocrinology and Reproduction Research Branch are directed at the elucidation of cellular mechanisms involved in hormone secretion and action, and at the investigation of normal and disordered function of the hypothalamic-pituitary system and its effects upon gonadal and adrenal function. These projects include studies on the characterization of peptide hormones and their cellular receptors; the structure-function relationships of peptide and glycoprotein hormones; the regulation of hormone biosynthesis and secretion; and the mechanisms of peptide hormone action in endocrine target cells. Of particular interest are the analysis of pituitary-gonadal and pituitary-adrenal regulation, the control of ovarian activity during the reproductive cycle and pregnancy, and the participation of hormone receptors in the regulation of pituitary, gonadal, and adrenal function. In the current year, research has been extended in several areas of hormone secretion and action, and on the receptor-mediated processes that are responsible for the control of steroid production in endocrine target cells. The role of hormones in cellular regulation has also been examined in selected areas of normal and disordered human endocrine function, and in appropriate animal model systems for the analysis of peptide secretion and the stimulatory and inhibitory control of target-cell function. The staff of the Branch share common interests in the secretion and mechanisms of action of peptide and glycoprotein hormones, the role of neuropeptides in hypothalamic-pituitary regulation, the control of gonadal and adrenal function by pituitary hormones, the renin-angiotensin system and aldosterone secretion, and the role of phosphorylation in metabolic regulation.

The section led by Kevin Catt undertakes research on the control of endocrine target cells by peptide hormones, in particular the characterization, regulation, and activation mechanisms of membrane receptors for gonadotropins, angiotensin II, gonadotropin-releasing hormone (GnRH), and corticotropin-releasing facotor (CRF). The receptor-mediated actions of gonadotropin-releasing hormone (GnRH) and other regulators of pituitary hormone secretion are studied in cultured anterior pituitary cells.

In the pituitary gland, GnRH receptors exhibit prominent variations in number during the ovarian cycle and after changes in steroid feedback, and are modulated by the rate of GnRH secretion from the hypothalamus. In cultured pituitary cells, GnRH receptors undergo down-regulation during exposure to GnRH agonists, followed by a subsequent increase in the receptor number that is dependent on protein synthesis. GnRH antagonists do not cause receptor down-regulation, but high affinity antagonist analogs bind for extended periods to cause receptor occlusion and prolonged inhibition of GnRH action. Analysis of the rat pituitary GnRH receptor by photoaffinity labeling has revealed two binding subunits of Mr 53,000 and 42,000. The receptor-activated processes leading to gonadotropin secretion are highly calcium dependent, and are initiated by rapid phospholipid hydrolysis with production of arachidonic acid metabolites, diacyl-glycerol, and inositol phosphates. The role of protein kinase C in gonadotrophin secretion is indicated by the ability of phorbol esters and synthetic diacylglycerols to stimulate LH release, and by the redistribution of protein kinase C between cytosol and membrane fractions in GnRH-stimulated gonadotrophs.

It is likely that the effects of arachidonate metabolites are integrated with those of calcium-calmodulin and calcium, phospholipid-dependent protein kinases during the immediate and sustained phases of GnRH-induced gonadotrophin secretion. Studies with dihydropyridine calcium channel agonist and antagonist derivatives, and measurements of cytoplasmic calcium in Quin-2-loaded cells, revealed that increases in intracellular calcium via voltage-sensitive calcium channels partially reproduce GnRH action, and suggest that GnRH causes activation of such channels in the gonadotroph. The increase in cytoplasmic calcium during GnRH action also originates in part from mobilization of internal calcium stores, and its relatively small magnitude is consistent with the concomitant activation of protein kinase C as an intermediate step in GnRH action.

The hormonal control of gonadal endocrine function by gonadotropins and growth factors was studied in ovarian and testicular target cells. The mechanisms of hormonal control of granulosa-cell maturation, and the production of specific receptors and other proteins that mediate hormone action in cells of the reproductive system, were analyzed in cultured granulosa cells. Studies with estrogen antagonists showed that the cAMP-mediated production of LH receptors in FSH-stimulated granulosa cells, demonstrated in earlier studies, was dependent upon the continued actions of estrogen throughout the maturation process. In addition, the ability of aromatase inhibitors to prevent LH receptor expression revealed that sustained production of estrogen by the maturing target cells was essential for FSH-induced granulosa cell differentiation. The inhibitory action of GnRH on ovarian function was found to be reproduced by activators of protein kinase C, indicating that this enzyme has a major role in GnRH action in the ovary as well as in the pituitary gland. In immature granulosa cells, phorbol esters suppressed FSH-induced aromatase activity and cellular aggregation and caused translocation of protein kinase C from cytosol to other cellular compartments. Synthetic diacylyglycerols also prevented FSH-induced LH receptor expression and progesterone production, but had less inhibitory effect than phorbol esters on cell aggregation and aromatase activity, and did not cause redistribution of protein kinase C. The prominent suppression of granulosa cell differentiation by phorbol esters reflects their rapid and prolonged action on protein kinase C. In contrast to such inhibitory effects in the immature granulosa cell, activation of protein kinase C in more differentiated cells caused stimulation of cyclic AMP formation and progesterone synthesis. Thus, in the mature granulosa cell, the calcium, phospholipid-dependent enzyme is also involved in the regulation of steroidogenesis by hormonal ligands including gonadotropins and GnRH.

The properties of angiotensin II (AII) receptors were studied in the adrenal zona glomerulosa, and the mechanisms leading to stimulation of steroidogenesis were analyzed in isolated glomerulosa cells from the rat and bovine adrenal cortex. AII receptors were further characterized by photoaffinity labeling with a C-terminal azido AII derivative, which possessed high labeling efficiency and was applied to the analysis of receptors in several target tissues. Isolation of the photolabeled AII receptor of the bovine adrenal gland was pursued by detergent solubilization and fractionation by ion exchange, lectin-affinity, and immunoaffinity chromatography. Studies on the actions of AII revealed that the calcium channel agonist, BAY K 8644, increased basal aldosterone production and enhanced the responses to AII and K^+ in a differential manner, by increasing the maximum aldosterone response to AII but not to K^+. These findings suggest that voltage-sensitive calcium channels are partially operative under basal conditions and are further activated by AII and

K^+. Elevation of cytoplasmic calcium by AII also depends upon mobilization of intracellular calcium stores by the products of ligand-stimulated phosphoinositide turnover. Microsomal receptors for the putative mediator of calcium mobilization, inositol-1,4,5-trisphosphate (IP_3) were identified in adrenal microsomes by binding studies with $[^{32}P]IP_3$, and show high specificity and affinity (Kd 5 nM) as well as low capacity for IP_3. The generation of inositol-1,4,5-trisphosphate from phosphatidylinositol bisphosphate during AII action was extremely rapid and was accompanied by significant production of IP_2 and inositol-4-monophosphate as well as formation of the inactive IP_3 isomer, inositol-1,3,4-trisphosphate. The finding that IP_3 is rapidly degraded to Ins-4-P contrasts with the previous view that Ins-1-P is the major metabolic product, and indicates that Ins-4-P serves as a marker of polyphosphoinositide turnover. The IP_3-receptor system and the activation of voltage sensitive calcium channels are the major mechanisms involved in the regulation of intracellular calcium by AII and potassium, respectively, in the adrenal zona glomerulosa cell.

The section directed by Greti Aguilera investigates the physiological and pathological aspects of the renin-angiotensin system, with emphasis on the role of AII in the regulation of aldosterone secretion and circulatory homeostasis. AII mediates the increases in aldosterone secretion and circulatory homeostasis. AII mediates the increases in aldosterone secretion during sodium restriction, but the adrenal effects of the peptide are dependent on the sensitivity of the glomerulosa cell to AII. In addition to the modulatory action of somatostatin and dopaminergic mechanisms as possible regulators of the adrenal sensitivity to AII, atrial natriuretic factor (ANF) is a potent inhibitor of aldosterone secretion. As well as exerting a preferential inhibitory effect on AII-stimulated aldosterone secretion in vitro, ANF was shown to antagonize the in vivo response of plasma aldosterone to AII infusion and sodium restriction, while ACTH-dependent aldosterone stimulation was unaffected. These actions of ANF provide further support for a role of the peptide in the regulation of adrenal responsiveness to AII. Studies on the mechanism of action of AII revealed a calcium/calmodulin protein kinase that phosphorylates a 100 kDa cytosolic protein in the adrenal glomerulosa zone. This enzyme is different from myosin light chain kinase and its activity was found to be regulated by AII. Previous studies demonstrating the presence of AII receptors in the rat brain were extended to the primate. In monkey brain, AII receptors were found in the circumventricular organs and other limbic structures, all related to the regulation of water intake, blood pressure and autonomic function. In the rat pituitary gland, AII stimulates prolactin secretion after binding to specific receptors located in the lactotrophs. In contrast to the adrenal and vascular AII receptors, pituitary receptors were unaffected by changes in sodium diet and plasma AII levels, but were regulated by estrogens. Incubation of pituitary cells with estradiol increased prolactin responses to AII but caused a time and dose-dependent decrease in AII receptors, indicating that the major regulatory effects of estrogens are exerted at post-receptor sites in the pituitary lactotroph.

Studies on the mechanisms of neuroendocrine regulation have focused on the actions of corticotropin releasing factor (CRF) receptors, and the interactions of CRF with other ACTH regulators including vasopressin (VP), angiotensin II (AII), norepinephrine and glucocorticoids. Previous studies revealed that the increases in plasma ACTH after adrenalectomy are accompanied by pituitary CRF

receptor down-regulation and desensitization. Further research in rats receiving CRF infusion has demonstrated that sustained exposure of the pituitary to CRF causes CRF receptor loss and a specific decrease in CRF-stimulated adenylate cyclase activity, which could partially account for the changes following adrenalectomy.

CRF receptors were previously demonstrated in the rat brain. Current studies in the monkey have shown the presence of CRF receptors in the cerebral cortex, limbic system, and related areas of the primate brain. In the peripheral nervous system, the importance of CRF receptors in the adrenal medulla was emphasized by studies in isolated bovine chromaffin cells which demonstrated the ability of CRF to stimulate catecholamine and met-enkephalin secretion. The interactions between ACTH regulators and their mechanism of action were analyzed in rat pituitary cells. In addition to the cyclic AMP-dependent mechanisms by which CRF stimulates the corticotroph, activators of protein kinase C such as phorbol esters, synthetic diacylglycerol and phospholipase C were found to stimulate ACTH secretion. This effect was additive to the stimulatory effect of CRF, but not to those of VP, AII and norepinephrine, suggesting the involvement of protein kinase C in the action of cyclic AMP-independent stimuli. In studies on glucocorticoid feedback, experiments in isolated pituitary cells demonstrated that the biphasic inhibitory pattern of ACTH secretion observed in vivo also occurs in vitro in the corticotroph. The two inhibitory components have different kinetics and sensitivity to corticosterone and probably involve different mechanisms of action of glucocorticoids in the corticotroph.

M. Dufau and her colleagues investigate the molecular basis of peptide hormone action, with particular emphasis on the characterization of gonadotropin receptors, activation of steroid biosynthesis in gonads and adrenal, and analysis of the biological activity of circulating gonadotropins. A major aspect of this program is concerned with the characterization of gonadal gonadotropin and prolactin receptors, and of the physical and functional relationships of the LH receptor site and adenylate cyclase. Microgram quantities of active lactogen receptors were purified from rat ovaries and subjected to structural and binding studies. The receptor is composed of two dissimilar subunits of Mr 88,000 and 40,000, the latter being probably an integral part of the larger form. Aggregation of receptor subunits and/or holoreceptor was suggested by FLPC fractionation of the free receptor in the presence of non-ionic detergents. Free receptors showed binding activity of Mrs 150,000 and 250,000, and aggregates dissociated upon SDS/mercaptoethanol treatment into the lower molecular forms. These could represent dimeric and trimeric forms of the holoreceptor (80,000). A method for iodination of purified prolactin receptor with preservation of binding activity was used to corroborate the Mr's of the free receptor and hormone-receptor complexes after crosslinking the ^{125}I-receptor with unlabeled hormone. The results confirmed values initially obtained by crosslinking experiments using labeled hormone and unlabeled receptor. When radiolabeled receptor was used for studies on receptor subunit aggregation, dimeric and trimeric forms were observed. These findings indicate that the detergent soluble receptor appears to contain aggregated forms of holoreceptors. The potential for aggregation of subunits and/or holoreceptors is suggested by these findings, and the mechanism by which aggregation or clustering of prolactin receptors would trigger the biological response remains to be elucidated. Chromatofocusing of free receptors showed three isoforms of pI 4.0, 5.0 and 5.3, indicating glycoprotein and/or phosphorylation heterogeneity.

The LH/hCG receptor was also purified, by sequential wheat germ lectin-Sepharose and hCG Sepharose chromatography, which also allows purification of lactogen receptor from the initial starting material. The LH receptor was identified as a single protein of Mr 70,000. This technique is simple and allows purification of microgram amounts of active receptor suitable for structural studies, microsequencing, and functional reconstitution studies.

The receptor-mediated actions of gonadotropins and other hormones were further analyzed in the testicular Leydig cell. The inability of the fetal/immature Leydig cell to be desensitized by gonadotropin, a characteristic of the adult cell, is due to the absence of an estradiol-mediated regulation of the androgen pathway (17 α-hydroxylase/17/20 demolase). In fetal rat Leydig cell cultures employed to analyze this differential response, estradiol caused an up-regulation of its receptor and an induction of the regulatory mechanism of the androgen pathway. The absence of this endogenous regulation in fetal life is due to very low levels of aromatase activity, undetectable estradiol production, and consequent low levels of estradiol receptor. The above explains the low levels of estradiol regulated 27K protein that is present in fetal cells and increased by E_2 treatment. This long-lived protein is undetectable post-natally and increases with age, and is present in the cytoplasmic matrix near the cisternae of rough and smooth endoplasmic reticulum. Future research with this system will help to clarify mechanisms responsible for emergence of the adult cell population. These cultures were also used for studies on GnRH agonists, which cause a lesion of microsomal enzymes that markedly inhibits steroid production. GnRH receptors were undetectable in fetal testes, appeared post-natally and increased with age. In fetal cultures, GnRH receptors were up-regulated by GnRH and reduced by LH treatment. Thus, GnRH-related peptides acting via GnRH receptors can influence the actions of LH on the fetal Leydig cell population. Opiate receptors and actions were found to be present in Sertoli cells. Opiate up-regulated its receptor and reduced FSH-stimulated androgen binding protein. Opioids of Leydig cell origin may modulate Sertoli cell function and influence the transport of testosterone to germinal cells. Testicular converting enzyme in mature rats was mainly associated with germinal cells (spermatids), results consistent with the effects of hormonal treatment. In contrast, the Leydig cells contain low quantities of converting enzyme, about 5% of the amount present in spermatids. Thus, locally produced angiotensin II could modulate the control of Leydig cell secretion and tubule function by gonadotropins.

The bioassay of serum LH and hCG was applied to further studies on the secretion of bioactive LH. The bioactivity of circulating LH appears to be rapidly modulated by gonadal steroids [i.e., normal men have higher B:I (bioactive:immunoreactive ratio) than cycling females; castration in rats decreases the B:I]. Also, the decrease in B:I of LH in castrated animals and differences in B:I observed in men vs. cycling females could be related to increased LH secretion and/or production rate. Previous estimates of LH blood production derived from immunoassay data markedly underestimated the quantity of bioactive LH secreted in man. The estimated bioactive blood production rate is about 30 fold higher than for LH immunoactivity, values that are in accord with the plasma B:1 ratio of 4 in normal men. The metabolic clearance rate for LH bioactivity was 33% less than that for immunoactivity. These results indicate that high endogenous production rates represent a predominant factor responsible for the high B:I ratio characteristic of plasma LH in normal men. In the female, as previously observed with testosterone pulsation in men, it is unlikely that a temporal relationship exists between individual bioactive LH and

pulses of progesterone secreted by the late corpus luteum. In both sexes, opioids slow GnRH pulses at the hypothalamic level and cause inhibition of LH secretion. The opiate antagonist, naltrexone, increases pulse frequency and mean integrated bioactive LH. Dissection of an early releasable LH pool by a single dose of IV naloxone could be of value to complement the GnRH test in physiopathological exploration of hypothalamic function in man. Steroids can modify GnRH secretion and estradiol replacement reinstates opioid suppression of bioactive LH secretion in orchidectomized patients with testicular feminization. Also, direct inhibitory effects of opioids on LH secretion, through high affinity pituitary receptors, could further modulate LH bioactivity. Using an in vitro lactogen bioassay, definite prolactin pulsations were detected at all stages of the cycle and B:I ratios were below or near unity. In contrast, serum B:I ratios were elevated during the rat proestrus surge. Prolactin biopotency is much higher in the rat than in the human and increases at proestrous, reflecting the physiological relevance of this hormone to luteal function in this species.

The section led by C. Strott investigates the physiology and regulation of adrenal steroidogenesis, focusing on the characterization of cellular steroid binding proteins and soluble factors which mediate steroidogenic responses to ACTH, and the analysis of cellular mechanisms of cholesterol utilization in steroid biosynthesis. The guinea pig, a cortisol-producing species, is employed as a model for studies on the regulation of steroidogenesis in the adrenal cortex, and the development and regulation of cellular zonation in the adrenal cortex.

The adrenal cortex of the guinea pig is composed of chromatically distinct outer and inner zones which can be separated by microdissection. In studies exploring the responsivity of the two zones to ACTH, these investigators have made the following observations: 1) There is no steroidogenic response to ACTH by cells isolated from the inner zone; 2) cholesterol side-chain cleavage activity (rate-limiting in steroidogenesis) is significantly lower in the inner zone and is not modulated by ACTH, stress, or chronic dexamethasone suppression; 3) the content of cholesterol is 3-4 times higher in the outer zone. Thus, the guinea pig presents an interesting animal model in which to investigate steroidogenesis, cholesterol metabolism, and the mechanism of action of ACTH by performing experiments on the two adrenocortical zones in a parallel fashion. The regulation of adrenocortical steroidogenesis by ACTH is complex and only partially understood. The accepted obligatory steps include: Stimulation of plasma membrane adenylate cyclase, increase in intracellular cAMP, and activation of cAMP-dependent protein kinase. The role of other kinases such as Ca^{2+}/calmodulin-and Ca^{2+}/phospholipid-dependent protein kinase is less clear. A large number of proteins (membranous and soluble) which are phosphorylated in response to ACTH have been reported. To date, however, no regulatory phosphoprotein has been identified. Phosphoprotein phosphatases have not been examined. Based on the use of inhibitors of protein and RNA synthesis, a critical role for protein synthesis in the stimulation of adrenal steroidogenesis by ACTH has been proposed. ' Adrenal steroid production is rapidly activated and deactivated (~2 min); such a process is considered too rapid to involve regulation at the level of translation. Regulation would, however, be compatible with protein modification. It is now well established that the covalent modification of protein is a crucial mechanism by which cellular processes are regulated. It is essential to identify and characterize the modified (e.g.,

phosphorylated or dephosphorylated) steroidogenic regulatory protein. This is the goal of the comparative approach, using the guinea pig adrenal cortex model.

The role of non-catalytic proteins in the adrenocortical steroidogenic process is uncertain. Non-catalytic proteins have been implicated in a variety of mechanisms such as regulation of cholesterol side-chain cleavage activity (the rate-limiting step in steroidogenesis), cholesterol and pregnenolone transport mechanisms, and secretory processes. However, no non-catalytic protein has yet been completely isolated and characterized in the adrenal cortex, and the elusive steroidogenic regulatory protein remains to be identified. The adrenal cortex of the guinea pig contains specific steroid-binding proteins which have been only partially purified and characterized; their function is as yet undetermined. For instance, there are proteins which specifically bind cholesterol, cholesteryl sulfate, pregnenolone, and pregnenolone sulfate. These proteins are of great interest because the rate-limiting reaction in steroidogenesis is the conversion of cholesterol to pregnenolone or cholesteryl sulfate to pregnenolone sulfate. The pregnenolone binding protein, which is isolated from the high speed soluble fraction, has been purified to the greatest extent, but the best current preparations still contain numerous contaminants. To purify the pregnenolone-binding protein further, two approaches are currently being used by this group: 1) Development of an affinity probe, 2) generation of antibodies to proteins electroeluted from sodium dodecyl sulfate gel slices. Several antisera have now been generated by Strott et al. which are presently being examined by Western blot analysis as well as for interaction with the pregnenolone-binding protein using the technique of sucrose density gradient analysis. It is planned in the near future to utilize high performance liquid chromatography for purifying isolated gel protein bands. In addition to purifying steroid-binding proteins, it is becoming increasingly apparent that it is necessary to isolate and characterize selected phosphoproteins. The latter task will be difficult and time-consuming, but necessary if a specific functional significance is to be ascertained for a particular phosphorylated (or dephosphorylated) protein.

H. C. Chen's section conducts research on the analysis, synthesis, and structure-function relationships of biologically active peptides and proteins. This work includes the identification and synthesis of unusual structures and sequences in amino acids and peptides, and the development of new techniques for peptide sequencing and synthesis.

A peptide sequence in proteins, -Pro-Lys-Lys-Lys-Arg-Lys-Val-, known to be associated with translocation of proteins into the nucleus, was synthesized as an N-trifluoroacetylated sulfohydroxysuccinimido ester. After coupling to avidin and removal of trifluoracetyl groups by piperidine, a conjugate was obtained that has two peptide units linked through the COOH-terminal group of the peptide to two ε-NH_2-groups in the tetrameric avidin molecule. This conjugate exhibited full biotin-binding activity. Similarly, a peptide of 22 amino acid residues, designated as Differentiation Peptide B, was synthesized by a solid phase method and derivatized as the $^{\alpha}N^{\varepsilon}$-trifluoroacetyl pentafluorophenyl ester, and then conjugated to bovine thyroglobulin. In both cases, defined linkages between peptide and protein were achieved.

Further research was performed on the biological properties of dimeric gonadotropin releasing hormone agonists and antagonists. Dimeric des-Gly^{10}-[D-Lys^{6}]-GnRH-NHE_t, cross-linked at the ε-amino group with -Gly_n-$COCH_2$-CO-Gly_n-(n=0, 1, 2), exhibited increased biological activities and mast cell histamine releasing activity as compared to the monomer. However, a dimer (n=1) was found to display a substantial increase in a post-coital antifertility effect as compared to the increase of histamine releasing side-effects. A similar approach was taken to the synthesis and purification of dimers of a GnRH antagonist of the following sequence: Ac-D-pC1Phe-D-pCLPhe-D-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH_2. Antisera against the agonist and the antagonist monomers were also generated.

In other sequencing studies, determination of the amino acid composition and partial amino acid sequence of induced NAD(P)H:menadione oxidoreductase allowed deduction of the correct reading frame of the cloned cDNA.

In work on the role of carbohydrate structures, Chen et al. examined the effect of such structures in human chorionic gonadotropin (hCG) on gonadotropic action and subunit association. Production and purification of hCG specific antibodies were also undertaken. The measurement of the rate of fluorescence enhancement of 1-anilinonaphthyl-8-sulfonate, when complexed with intact or HF-deglycosylated hCG, revealed that the subunits of deglycosylated hCG reassociated nearly 10-fold faster than unmodified subunits. Studies on the reassociation rate of hybrid subunits from the intact β-subunit of ovine luteinizing hormone and subunits of intact and modified hCG suggest that carbohydrate moieties in the α-subunit dominate the reassociation behavior. Antibodies raised against HF-deglycosylated hCG, and a bivalent $F(ab')_2$ fragment prepared and purified from a sequence-specific anti-carboxymethylated hCGβ antibody, were shown to enhance stimulation of cAMP and progesterone production in rat differentiated granulosa cells preincubated with the modified hCG. These findings are consistent with the notion that hormonal activation may be caused by cross-linking and/or microaggregation of the HF-hCG:receptor complex. However, antibodies which bind to an appendage of HF-hCG which is too flexible and distant from the complex, or which have the ability to strip off hormone from the receptor complex, were ineffective in eliciting activation.

The section directed by K. P. Huang studies the regulation and hormonal control of glycogen metabolism in normal and diabetic tissues, and the activities of glycogen synthase, phosphorylase kinase, and protein kinase C. Phosphorylation-dephosphorylation of proteins is one of the most important mechanisms for the regulation of cellular functions. Protein kinase C, a Ca^{2+}/phospholipid-dependent protein kinase, has emerged as a pivotal regulatory element for cell growth, differentiation, gene expression, hormone secretion, cell surface receptor function, and cellular metabolism. This protein kinase can be activated by diacylglycerol, a second messenger generated by "signal"-induced breakdown of phosphoinositides. In addition, protein kinase C has been identified as a receptor for tumor-promoting phorbol esters, which elicit pleiotropic responses comparable to those induced by many hormones and growth factors. During the past year, three isozymic forms of rat brain protein kinase C have been purified to near homogeneity. Polyclonal and monoclonal antibodies against these enzymes were prepared to permit an immunochemical characterization. These isozymes are distinguishable by peptide mapping and immunological analysis, indicating that they are products of different genes. The various protein kinase C isozymes were found to be enriched in different regions of rat

brain, and expressed differentially during brain development. Activation of protein kinase C in cells was studied by using EL4 thymoma cells which can be induced to secrete interleukin-2 in response to tumor-promoting phorbol esters. These compounds promote the translocation of protein kinase C from cytosol to the particulate fraction, and a rapid degradation of the enzyme. The phorbol ester-induced translocation and degradation of protein kinase C may be early events in the secretion of interleukin-2. In vitro, tryptic degradation of purified protein kinase C generates forms of the kinase and phorbol ester-binding protein that are active in the absence of Ca^{2+}. The structure-function relationships of protein kinase C were investigated by using monoclonal antibody specific for the phorbol ester-binding domain. This antibody reduces the binding of phorbol ester to the enzyme without inhibiting the kinase activity. Finally, protein kinase C activity was shown to be regulated by autophosphorylation, and the resulting phosphorylated kinase exhibited higher affinity for Ca^{2+} and phorbol esters than the non-phosphorylated enzyme.

Endocrinology and Reproduction Research Branch

Chief, *Kevin J. Catt, M.D., Ph.D.

Senior Staff

*Greti Aguilera, M.D.
*Hao-Chia Chen Ph.D.
*Maria L. Dufau, M.D., Ph.D.
*Kuo-Ping Huang, Ph.D.
*Charles A. Strott, M.D.

Fellows

Mohammad Anwar Goheer, Ph.D.
Michael L. Knecht, Ph.D.
Young Ja Lee, Ph.D.
Monica Millan, M.D.

Visiting Program

Tamas Balla, M.D.
Pei Feng, M.D.
Azra Khanum, Ph.D.
Mitsumasa Kubo, M.D., Ph.D.
Satoshi Kusuda, M.D., Ph.D.
Takashi Minegishi, Ph.D.
Reginald Morgan, Ph.D.
Hiroki Nakabayashi, M.D.
Shigeru Nakano, M.D., Ph.D.
Keiichi Tasaka, M.D.
Yasuyoshi Yoshida, M.D.
Moshe Zilberstein, M.D.

Experts

Freesia Huang, Ph.D.

Guest Researchers

Michael Blank, Ph.D.
Mary Carson, Ph.D.
John P. Chang, Ph.D.
Chandana Das, Ph.D.
John J. Evans, Ph.D.
Fakhrozaman Ghazanfari, Ph.D.
Gaetan Guillemette, Ph.D.
Jennifer Larson, M.D.
Rosalind McIntosh, Ph.D.
Maria Nishihara, M.D.
Karl Sebok, Ph.D.
Stanko Stojilkovic, Ph.D.

Bibliography

Abou Samra AB, Catt KJ, Aguilera G. Biphasic inhibition of ACTH release by corticosterone in cultured anterior pituitary cells. Endocrinology 1986;119:972-7.

Abou-Samra AB, Catt KJ, Aguilera G. Involvement of protein kinase C in the regulation of adrenocorticotropin release from rat anterior pituitary cells. Endocrinology 1986;118:212-7.

Abou-Samra AB, Catt KJ, Aguilera G. Role of arachidonic acid in the regulation of adrenocorticotropin release from rat anterior pituitary cell cultures. Endocrinology 1986;119:1427-31.

Aguilera G, Catt, KJ. Participation of voltage-dependent calcium channels in the regulation of adrenal glomerulosa function by angiotensin II and potassium. Endocrinology 1986;118:112-8.

Aguilera G, Catt KJ. Regulation of the sensitivity of the adrenal glomerulosa cell during altered sodium intake. In: Mantero E, Biglieri EG, Funder JW, Scoggins BA, eds. The Adrenal Gland and Hypertension. NY: Raven Press, 1986;33-53.

Aguilera G, Wynn PC, Harwood JP, Hauger RL, Millan MA, Grewe C, Catt KJ. Receptor-mediated actions of corticotropin-releasing factor in pituitary gland and nervous system. Neuroendocrinology 1986;43:79-88.

Baukal AJ, Guillemette G, Rubin R, Spat A, Catt KJ. Binding sites for inositol trisphosphate in the bovine adrenal cortex. Biochem Biophys Res Commun 1986;133:532-8.

Beitins IS, Dufau ML. Pulsatile secretion of progesterone from the human corpus luteum poor correlation with bioactive LH pulses. Acta Endocrinol. 1986;111:553-7.

Blank MS, Chin M, Dufau ML. Bioactivity of serum and pituitary prolactin during the rat estrous cycle. Endocrinology 1986; 86:1886-91.

Blank MS, Dufau ML. Definition of bioactive pulsations during the menstrual cycle. Clin Endocrinol 1986;24:1-7.

Blank MS, Fabbri A, Catt KJ, Dufau ML. Direct inhibition of gonadotroph function by opiates. Trans Assoc Am Physicians 1985;XCVII:1-9.

Blank MS, Fabbri A, Catt KJ, Dufau ML. Inhibition of luteinizing hormone release by morphine and endogenous opiates in cultured pituitary cells. Endocrinology 1986;118:2097-2101.

Catt KJ, Aguilera G. Control of aldosterone secretion. In: New MI, Borrelli P, eds. Dexamethasone-suppressible hyperaldosteronism. NY: Raven Press, 1986;139-149.

Catt KJ, Dufau ML, eds. Hormone action and testicular function. New York:Annals of the New York Academy of Sciences, 1984;708.

Catt KJ, Loumaye E, Wynn PC, Iwashita M, Hirota K, Morgan RO, Chang JP. GnRH receptors and actions in the control of reproductive function. J Steroid Biochem 1985;23:677-89.

Catt KJ, Pierce JG. Gonadotropic hormones of the adenohypophysis. In: Yen S, Jaffe R, eds. Reproductive endocrinology: physiology, pathology and clinical management. Philadelphia: W B Saunders, 1986;75-114.

Catt KJ, Wynn PC, Millan MA, Mendelsohn FAO, Aguilera G. Brain Receptors for angiotensin II and corticotropin-releasing factor. In: Ganong WF, Martini L, eds. Frontiers in neuroendocrinology. NY: Raven Press, 1986;225-53.

Chan KFJ, Stoner GL, Hashim GA, Huang KP. Substrate specificity of rat brain calcium-activated and phospholipid-dependent protein kinase. Biochem Biophys Res Commun 1986;134:1358-64.

Chang JP, Graeter J, Catt KJ. Coordinate actions of arachidonic acid and protein kinase C in gonadotropin-releasing hormone-stimulated secretion of luteinizing hormone. Biochem Biophys Res Commun 1986;134:134-9.

Chang JP, McCoy EE, Greater J, Catt KJ. Participation of voltage-dependent calcium channels in the action of gonadotropin-releasing hormone. J Biol Chem 1986;261:9105-8.

Childs GV, Morell JL, Niendorf, A, Aguilera G. Cytochemical studies of CRF receptors in anterior lobe corticotropes: Binding glucocorticoid regulation and endocytosis of (Biotinyl-Ser^{1}1) CRF. Endocrinology 1986;119:2129-42.

Ciocca D, Winters C, Dufau ML. Expression of an estrogen regulated protein in rat testis Leydig cells. J Steroid Biochem 1986;24:219-29.

Darbon JM, Knecht M, Ranta T, Dufau ML, Catt KJ. Follicle-stimulating hormone and gonadotropin-releasing hormone regulate cyclic AMP-dependent protein kinase in cultured ovarian granulosa cells. In: Ryan RJ, Toft DO, eds. Proceedings of the Fifth Ovarian Workshop. IL: Ovarian Workshops, 1985;403-8.

Dufau ML, Knox GF. Fetal Leydig cell culture an in vitro system for the study of trophic hormone and GnRH receptors and actions. J Steroid Biochem 1985;23:743-55.

Fabbri A, Tsai-Morris CH, Luna S, Fraioli F, Dufau M. Opiate receptors are present in the rat testis. Identification and localization in Sertoli cells. Endocrinology 1985;117:2544-6.

Feng P, Catt KJ, Knecht M. Transforming growth factor-beta regulates the inhibitory actions of epidermal growth factor during granulosa cell differentiation. J Biol Chem 1986;261:14167-70.

Fraioli F, Fabbri A, Gnessi L, Moretti C, Bonifacio V, Ferreti R, Isidori A, Dufau ML. Naloxone increases bioactive LH in man: evidence for selective release of an early LH pool. J Endocrinol Invest 1986;8:513-7.

Hausdorff WP, Aguilera G, Catt KJ. Selective enhancement of angiotensin II-and potassium-stimulated aldosterone secretion by the calcium channel agonist BAYK 8644. Endocrinology 1986;118:869-74.

Hirota K, Hirota T, Aguilera G, Catt KJ. Gonadotropin release and of calcium activated phospholipid-dependent protein kinase in phorbol stimulated rat pituitary cells. Arch Biochem Biophys 1986;249:557-62.

Holmes MC, Antoni FA, Aguilera G, Catt KJ. Magnocellular axons in passage through the median eminence release vasopressin. Nature 1986;319:326-9.

Holmes MC, Antoni FA, Catt KJ, Aguilera G. Predominant release of vasopressin vs corticotropin releasing factor from the isolated median eminence after adrenalectomy. Neuroendocrinology 1986;43:245-55.

Huang K-P, Chan K-FJ, Singh TJ, Nakabayashi H, Huang FLL. Autophosphorylation of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase. J Biol Chem 1986;261:12134-40.

Huang, K-P, Huang FL. Conversion of protein kinase C from a Ca^{2+}-dependent to an independent form of phorbol ester-binding protein by digestion with trypsin. Biochem Biophys Res Commun 1986;139:320-6.

Huang K-P, Huang FL. Immunochemical characterization of rat brain protein kinase C. J Biol Chem 1986;31:14781-7.

Huhtaniemi IT, Rajaniemi HJ, and Catt KJ. Kinetic and autoradiographic studies on binding of hCG to the testicular LH receptors of neonatal rats. J. Reprod Fertil 1986;78:73-82.

Inese Z, Dufau M. Pulsatile secretion of progesterone from the human corpus luteum: poor correlation with bioactive LH pulses. Acta Endocrinol 1986;111:553-7.

Iwashita M, Catt KJ. Phorbol ester-stimulated LH release is enhanced by calmodulin antagonist and inhibited by a GnRH antagonist. Endocrinology 1985;117:738-46.

Iwashita M, Evans MI, Catt KJ. Characterization of a gonadotropin-releasing hormone receptor site in term placenta and chorionic villi. J Clin Endocrinol Metab 1986;62:127-33.

Khanum A, Dufau ML. Inhibitory action of forskolin on adenylate cyclase activity and cyclic AMP genration. J Biol Chem 1986;261:11456-9.

Knecht M. Production of a cell-associated and secreted plasminogen activator by cultured rat granulosa cells. Endocrinology 1986;118:348-53.

Knecht M, Feng P, and Catt J. Transforming growth factor-beta regulates the expression of luteinizing hormone receptors in ovarian granulosa cells. Biochem Biophy Res Commun 1986;139:800-7.

Knecht M, Ranta T, Feng P, Shinohara O, Catt KJ. GnRH as a modulator of ovarian function. J Steroid Biochem 1985;23:771-8.

Knecht M, Ranta T, Shinohara O, Hoffmann ST, Catt KJ. Cyclic GMP accumulation in cultured rat granulosa cells is due to the inhibition of phosphodiesterase activity by cyclic AMP-inducing ligands. In: Ryan RJ, Toft DO, eds. Proceedings of the Fifth Ovarian Workshop. IL: Ovarian Workshops, 1985;95-9.

Knecht M, Shinohara O, and Catt KJ. Biosynthesis of cellular and secreted proteins during follicle-stimulating hormone-induced granulosa cell differentiation. Endocrinology 1986;119:1388-96

Lee KO, Gesundheit N, Chen HC, and Weintraub BD. Enzymatic deglycosylation of thyroid-stimulating hormone with peptide N-glycosidase F and endo-beta-N-acetylglucosaminidase F. Biochem Biophys Res Commun 1986;138:230-7.

Mendelsohn FAO, Dunbar M, Allen A, Chou ST, Millan M, Aguilera G, Catt KJ. Angiotensin receptors in the kidney. Federation Proceedings 1986;45:1420-5.

Mikami K, Strott CA. Cyclic AMP-dependent protein kinase activity and protein phosphorylation in zones of the adrenal cortex. Biochem Biophys Research Commun 1986;138:895-901.

Millan M, Aguilera G, Wynn P, Mendelsohn F, Catt KJ. Autoradiographic localization of brain receptors for peptide hormones: angiotensin II, corticotropin-releasing factor and gonadotropin releasing factor. Methods Enzymol 1986;124:590-606.

Millan MA, Jacobowitz DM, Hauger RL, Catt KJ. Distribution of corticotropin-releasing factor receptors in primate brain. Proc Nat Acad Sci 1986;83:1921-5.

Mitani M, Dufau ML. Purification and characterization of prolactin receptors from rat ovary. J Biol Chem 1986;261:1309-15.

Morell JL, Brown J. Solid phase synthesis of ovine corticotropin releasing factor. Int J Pept Protein Res 1985;26:49-54.

Naor Z, Kiesel L, Vanderhoek JY, Catt KJ. Mechanism of action of gonadotropin releasing hormone: Role of lipoxygenase products of arachidonic acid in luteinizing hormone release. J Steroid Biochem 1985;23:711-7.

Nonomura K, Obara T, Strott CA. Low density lipoprotein receptor activity in the guinea pig adrenal cortex. I. Zonal characterization and response to adrenocorticotropin. Endocrinology 1986; 118:653-60.

Nonomura K, Strott CA. Low density lipoprotein receptor activity in the ethinylestradiol. Biochim Biophys Acta 1986;876:264-70.

Platia MP, Krudy AG, Transvaginal fluoroscopic recanalization of a proximally occluded oviduct. Fertil Steril 1985;44:704-6.

Ranta T, Knecht M, Darbon JM, Baukal AJ, Catt KJ. Calcium-mediated inhibition of follicle-stimulating hormone-induced granulosa cell differentiation by gonadotropin-releasing hormone. In: Ryan RJ, Toft DO, eds. Proceedings of the Fifth Ovarian Workshop. IL: Ovarian Workshops, 1985;125-30.

Shinohara O, Knecht M, Catt KJ. Calcium- and phospholipid-dependent protein kinase activity and substrate phosphorylation in the rat ovary. In Ryan RJ, Toft DO, eds. Proceedings of the Fifth Ovarian Workshop. IL: Ovarian Workshops, 1985;443-8.

Shinohara O, Knecht M, Catt KJ. Differential actions of phorbol ester and diacylglycerol on inhibition of granulosa cell maturation. Biochem Biophys Res Commun 1985;133:468-74.

Shinohara O, Knecht M, Catt J K. Inhibition of gonadotropin induced granulosa cell differentiation by activation of protein kinase C. Proc Nat Acad Sci 1985;82:8518-22.

Shinohara O, Knecht M, Feng P, Catt KJ. Activation of protein kinase C potentiates cyclic AMP production and stimulates steroidogenesis in differentiated ovarian granulosa cells. J Steroid Biochem 1986;24:161-8.

Suarez-Quian CA, Wynn PC, Catt KJ. Receptor-mediated endocytosis of GnRH analogs: differential processing of gold-labeled agonist and antagonist derivatives. J Steroid Biochem 1986;24:183-92.

Tsai-Morris C-H, Knox GF, Luna S, Dufau ML. Acquisition of estradiol-mediated regulatory mechanism of steroidogenesis in cultured fetal rat leydig cells. J Biol Chem 1986;261:3471-4.

Udelsman R, Harwood JP, Millan MA, Chrousos GP, Goldstein DS, Zimlichman R, Catt KJ, Aguilera G. Functional corticotropin releasing factor receptors in the primate peripheral sympathetic nervous system. Nature 1986;319:147-50.

Veldhuis JD, Fraioli F, Rogol A, Dufau ML. Metabolic disposition of biologically active luteinizing hormone in man. J Clin Invest 1986;77:1122-8.

Veldhuis JD, Rogol AD, Peres-Palacios, Stumpf, G, Kitchin JD, Dufau ML. Endogenous opiates participate in the regulation of pulsatile luteinizing hormone release in an unopposed estrogen milieu: studies in estrogen-replaced, gonadectomized patients with testicular feminization. J Clin Endocrinol Metab 1985;61:790-3.

Wortsman J, Moses HW, Dufau ML. Increased incidence of thyroid disease among men with hypergonadotropic hypogonadism. Am J Med 1986;80:1055-9.

Wynn PC, Suarez-Quian CA, Childs GV, Catt KJ. Pituitary binding and internalization of radioiodinated GnRH agonist and antagonist ligands in vitro and in vivo. Endocrinology 1986;119:1852-63.

Laboratories of the Scientific Director--

° Section on Viruses and Cellular Biology
Arthur S. Levine, M.D., Head

Determining the mechanisms that maintain the integrity of the genome, but permit cellular proliferation and differentiation, is basic to an understanding of the development of multi-cellular organisms. One approach to investigating these regulatory mechanisms is to study the behavior of DNA viruses (e.g., SV40 and adenovirus) which infect and transform eukaryotic cells; the viruses themselves model many aspects of eukaryotic gene structure and regulation.

Rodent cells transformed by DNA viruses are of particular interest in that all such cells display the same phenotypic characteristics in vitro (e.g., high proliferative rate, serum independence), yet only the cells transformed by certain DNA viruses (e.g., SV40) readily induce tumors when inoculated in vivo. H. Haddada and A. S. Levine, in collaboration with A. M. Lewis (NIAID), have questioned whether this important difference is due to the level of Class I MHC antigens expressed on the cell surface--with some, but not all, DNA viruses inducing a down-regulation of these antigens. (Cells lacking a display of MHC antigens could evade the host's immune defenses and be highly tumorigenic.) These workers, employing a large number of transformed rodent cell lines, have now found that the tumorigenicity of rodent cells transformed by various DNA viruses cannot be correlated with the level of Class I MHC antigen expression. With this result in hand, these investigators have sought another explanation for the difference in oncogenicity between non-oncogenic and highly oncogenic DNA viruses. K. Akagi, A. S. Levine, and C. T. Patch find that oncogenic and non-oncogenic transformed cells secrete very similar TGF α-like mitogenic factors. However SV40-transformed hamster cells (which are highly oncogenic in vivo), but not adenovirus-2 transformed cells (which are non-oncogenic), also secrete a powerful mitogenic inhibitor. The inhibitor, which has a MW of 24 kD, inhibits thymidine uptake by con A-stimulated spleen lymphocytes, suggesting that it might contribute to the extreme oncogenicity of SV40-transformed cells by inhibiting mobilization of immune effector cells at the site of tumor cell proliferation.

In other work, B. J. Matthews, A. S. Levine, and K. Dixon found that deletion mutations in the small t-antigen gene alter the spectrum of tumors induced by SV40: Subcutaneously injected small t-antigen mutants of this virus often induce abdominal lymphomas and osteosarcomas in hamsters, rather than the subcutaneous fibro-sarcomas induced by wild-type SV40. Previous studies have suggested that a competent small t protein is required for the in vitro transformation of stationary cells, perhaps acting as a growth factor. These new results suggest that in the absence of this putative factor, the mutant viruses may preferentially transform rapidly proliferating cells (e.g., lymphoblasts, osteoblasts).

K. Dixon, J. Hauser, A. S. Levine, et al. are also studying the mechanism of mutagenesis, using an SV40-based shuttle vector as a probe to investigate the molecular mechanisms by which DNA-damaging agents induce mutations in mammalian cells. The shuttle vector (pZ189) allows these workers to induce mutations in a mammalian system, identify them in a bacterial system, and then sequence the mutant DNA. The vector contains the SV40 replication origin and early region

so that it replicates like SV40 virus in mammalian cells. The vector also contains the PBR327 bacterial plasmid origin and replication functions so that it can replicate in bacterial cells. In addition, pZ189 carries the gene for ampicillin resistance to provide a selectable phenotype in bacterial cells, and the gene for the $Su3^+$ suppressor tRNA to provide a well-defined target for mutagenesis.

By passing the shuttle vector sequentially through monkey cells and E. Coli (with or without first damaging the vector DNA in vitro), this group has analyzed both spontaneous and UV-induced mutations (the spontaneous mutations presumably arise during repair of the vector DNA, damaged by nucleases during transfection). Analysis of the spontaneous point mutations revealed a striking sequence specificity in mutation induction, suggesting that the DNA polymerase preferentially misincorporates A opposite C; the frequency of this event is strongly influenced by the neighboring nucleotides. In collaboration with P. Munson (LTPB), mathematical modeling and statistical analysis were used to demonstrate that there is a good correlation between the sites and frequencies of UV-induced mutations and the sites and frequencies of UV-induced DNA damage. In another collaborative project (with M. Protic-Sabljic, NCI), these investigators demonstrated that the pyrimidine cyclobutane dimer is the major UV-induced photoproduct responsible for mutagenesis in the shuttle vector system.

Studies on mutation induction with the shuttle vector system are being extended in three different directions. First, these workers seek to determine what role inducible cellular responses may play in mutagenesis (a pathway that would be analogous to the "SOS" response in bacteria). They have shown that when cells are treated with carcinogens prior to transfection with the vector, the frequencies of both spontaneous and UV-induced mutations increase. They also find that there is an increase in the excision repair capacity of mammalian cells following carcinogen treatment. Secondly, in collaboration with D. Nebert et al. (LDP), this group is utilizing cell lines that differ in their expression of P450 genes, and therefore in their ability to metabolize benzo[a]pyrene to the ultimate carcinogen (benzopyrenediolepoxide), in order to test directly the role of carcinogen metabolism on mutation induction. Finally, these investigators have begun to use a mammalian cell-derived in vitro DNA replication system to study more precisely the steps in mutation induction. They have so far demonstrated, using the pZ189 shuttle vector, that replication fidelity is surprisingly high in this in vitro system. All of this work, taken together, is significant in that it promises to offer considerable insight into the question of how DNA damaging agents interfere with normal DNA replication and induce mutations in mammalian cells; mutations are the underlying cause of most inherited diseases and many developmental abnormalities. Moreover, much recent evidence supports the notion that it is point mutation of normal cellular oncogenes which ultimately leads to cellular transformation and cancer.

Finally, B. J. Matthews, A. S. Levine, and K. Dixon have studied the process of DNA damage-induced amplification of viral sequences in mammalian cells. (Gene amplification is an important mechanism, in addition to mutagenesis, that undermines the stability of the genome.) This group studied a series of SV40-induced hamster tumor cell lines that differ in their capacity to amplify viral sequences. They found that several of their cell lines were unable to support carcinogen-induced amplification of the viral sequences although these lines were not defective in carcinogen-induced amplification of cellular genes. The

reason for this difference appears to lie in the limiting molecular anatomy of the viral as opposed to cellular genes (i.e., the presence or absence of tandem DNA duplication, which facilitates excision and consequently amplification). This restriction in the cell lines that did not support viral amplification could be overcome by fusion of the hamster cells with monkey cells, suggesting that differences in the cellular milieu may have a profound effect on the process of gene amplification.

Because of the mounting evidence that tumorigenesis, mutagenesis, and normal events in developmental biology are intimately related, this section's recent work should help to illuminate the mechanisms which regulate basic developmental phenomena.

° Section on Growth Factors--
Gordon Guroff, Ph.D., Head

This section is studying the mechanism of action of growth factors of neurobiological interest, and especially, the physiology of nerve growth factor (NGF). NGF is one of the best characterized of the large and increasing number of growth and differentiation factors already known. NGF is required for the survival and ultimate development of the sympathetic and sensory nervous systems and as well, it has some action on the adrenal medulla, the central nervous system, and on tumors arising from cells derived from the neural crest. A fuller knowledge of the mechanism of action of NGF should lead to a better understanding of the mode of regulation of the growth and differentiation of neurons. NGF is of particular interest also in that it may be involved in the regrowth of damaged or severed neural tracts, an issue of considerable clinical significance.

Nerve growth factor, a polypeptide, appears to control the expression of specific genes in target neurons, and thus to influence the development and differentiation of these cells. Guroff and his colleagues have been studying the intracellular events which follow the cell's exposure to NGF, using PC12, a pheochromocytoma cell which differentiates in response to NGF. Using this system, their goal has been to specify the cascade of intracellular biochemical events initiated by the binding of NGF to its receptor. Secondly, they are attempting to understand how that signal, after being received in the nucleus, is transduced into changes in the DNA that alter the transcription of specific genes. Thirdly, these investigators hope to discover what genes are expressed and which are repressed, such that nerve growth factor alters the phenotype of its target cells.

In studies utilizing PC12, Guroff et al. have now found that one of the earliest events after NGF binding is an increase in phosphoinositide metabolism, an event usually correlated with activation of the calcium and phospholipid-dependent enzyme, protein kinase C. This activation appears to be one of several changes in kinase function that occur, in some cases sequentially, within the PC12 cells. These workers have developed a cell-free, soluble phosphorylation system which reflects the prior treatment of the cells with NGF, a system which involves the phosphorylation of a protein of 100 kD (Nsp100). In extracts from cells treated with NGF, the phosphorylation of Nsp100 is decreased, and this decrease is caused by phosphorylation of Nsp100 kinase by protein kinase C. Guroff et al. have prepared two other cell-free

phosphorylation systems that also reflect the actions of NGF, one ribosomal and one nuclear. They have found that a different phosphorylation pathway, involving a cAMP-dependent kinase, leads to NGF-induced increases in the phosphorylation of the S6 protein of the ribosomes. The pattern that is emerging is that NGF activates a series of cellular kinases leading to the phosphorylation of some key cellular proteins. One of these proteins is found in the nucleus, and its phosphorylation may underlie changes in gene expression.

With regard to how the intracellular signal stimulated by NGF is transduced into changes in DNA, Guroff et al. have found that changes in nuclease sensitivity of the DNA from NGF-treated cells are paralleled by changes in DNA morphology (i.e., the chromatin becomes condensed). This finding suggests that the overall number of genes being transcribed in cells that are differentiated is lower than the number in cells that are rapidly dividing.

Several years ago, Guroff and his coworkers found that one consequence of NGF treatment of PC12 cells is an apparent decrease in the display of EGF receptors, such that as the nerve cell begins to differentiate under the influence of NGF, it loses its sensitivity to the proliferative stimulus of EGF. They have now shown that the decreased binding of EGF to NGF-treated PC12 cells actually represents a decrease in the receptor number, and not a change in the properties of existing receptors. Currently, these workers are investigating the transcription of the EGF receptor gene in NGF-treated cells. If, in fact, they are able to demonstrate that regulation of the EGF receptor is at the transcriptional level, they would have in hand one of the few systems with which to study the actions of NGF on the transcription of a specific gene.

Section on Viruses and Cellular Biology

Head, Arthur S. Levine, M.D.

Senior Staff

Cephas T. Patch, Ph.D.

Fellows

Kathleen Dixon, Ph.D.

Visiting Program

Michael Carty, Ph.D.
Mohamed Hedi Haddada, Ph.D.
Koichiro Murai, M.D.
Emmanuel Roilides, M.D.

Section on Growth Factors

Head, Gordon Guroff, Ph.D.

Fellows

Margarita Contreras, Ph.D.

Visiting Program

Tokiko Hama, Ph.D.
Shinichi Koizimi, Ph.D.
Philip Lazarovici, Ph.D.

Guest Researchers

Frank Collins, Ph.D.
Ko Fujita, M.D.

Bibliography

Greiner C, Lloyd AT, Guroff G. Ontogeny of the nerve growth factor receptor in primary cultures of neural crest cells. Dev Brain Res 1986;26:145-50.

Haddada H, Lewis AM, Sogn JA, Coligan JE, Cook JL, Walker TA, Levine AS. Tumorigenicity of hamster and mouse cells transformed by adenovirus type 2 and 5 is not influenced by the level of class I major histocompatibility antigens expressed on the cells. Proc Natl Acad Sci 1986;83:9684-8.

Hama T, Huang KP, Guroff G. Protein kinase C as a component of a nerve growth factor-sensitive phosphorylation system in PC12 cells. Proc Natl Acad Sci 1986;83:2353-7.

Hashimoto S, Iwasaki C, Kuzuya H, Guroff G. Regulation of nerve growth factor action on Nsp100 phosphorylation in PC12 cells by calcium. J Neurochem 1986;46:1599-1604.

Hauser J, Seidman MM, Sidur K, Dixon K. Sequence specificity of point mutations induced during passage of a UV-irradiated shuttle vector plasmid in monkey cells. Mol Cell Biol 1985;6:277-85.

Protic-Sabljic M, Tuteja N, Munson PJ, Hauser J, Kraemer KH, Dixon K. UV-induced cyclobutane pyrimidine dimers are mutagenic in mammalian cells. Mol Cell Biol 1986;6:3349-56.

Yavin E, Hama T, Gil S, Guroff G. Nerve growth factor and gangliosides stimulate the release of glycoproteins from PC12 phenochromocytoma cells. J Neurochem 1986;46:794-803.

NIH Library, Building 10
National Institutes of Health
Bethesda, Md. 20892

LIBRARY
NIH
Amazing Research.
Amazing Help.

http://nihlibrary.nih.gov

10 Center Drive
Bethesda, MD 20892-1150
301-496-1080

AUG 1987

INFORMATION CONSERVATION INC.

NIH LIBRARY
3 1496 00324 8237