



NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

ANNUAL REPORTS

DIVISION OF INTRAMURAL RESEARCH

October 1, 1987 to September 30, 1988

PREFACE

The National Institute of Diabetes and Digestive and Kidney Diseases is responsible for research on a wide spectrum of diseases which are among the most common, disabling, chronic, and costly that afflict our population. They affect over 30 percent of the Nation, exact an enormous toll in terms of human suffering, and their economic impact exceeds \$75 billion annually.

This report reflects the activities and advances of our intramural research program during the past year--a program of which we are justly proud. Our staff, in basic or clinical research, is widely acknowledged to be a highly innovative and productive group of scientists. It is also an intellectually flexible one which seems to pursue biological phenomena and scientific areas located most closely to the moving edge of contemporary research. We have moved the frontline of our investigations from the level of the whole organ to the cellular, the molecular and even submolecular levels to pursue the most basic life processes and elucidate the mechanisms that control them. But not all are mapping or cloning genes. Others are pursuing clinical aspects of diabetes, endocrine, digestive, nutritional and kidney diseases. We are proud of our intramural tradition of training--be it younger scientists or visitors from elsewhere. It is a mutually enriching process that tends to make a Fountainhead of Excellence from what might otherwise remain an Island of Excellence, and we are proud of the alumni of our intramural program that are now prominent faculty members at universities throughout the country.

The pages that follow describe ongoing studies, contemporary achievements, and plans for the future. We hope that this report for FY 1988 will be as informative and gratifying to its readers, as its predecessors.

Phillip Gorden, M.D.
Director
National Institute of
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PROJECT NUMBERS

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASE

ACTIVE PROJECTS

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Annual Report of the
Mathematical Research Branch

National Institute of Diabetes and Digestive and Kidney Diseases

Current research projects of the Mathematical Research Branch reflect a broad range of interests in the development and application of theoretical models as well as quantitative methodologies to biological systems.

This research involves several different collaborations within the Branch and with other research groups, both at the NIH and elsewhere. This report describes recent work in the areas of molecular biology, synaptic neurobiology, electrical oscillations in nerve and secretory cells, auditory physiology, cell energetics, renal physiology, and microcirculation and facilitated transport.

Synaptic Neurobiology

Dendritic spines. Work has continued on a biophysical theory to describe neuronal integrative properties which involve large numbers of dendritic spines. We have applied our new cable theory for continuously distributed spines to compare in more detail the effectiveness for initiating spread of activity when excitable channels are located either in the spine heads or on the dendritic shaft. Fewer synapses are needed to start propagation when the channels are in the heads, and the minimal number of synapses for initiation is less dependent on spine stem resistance when the channels are in the shaft. With channels in the head, an individual spine contributes more sensitively to the success or failure of propagation and therefore may play a significant role in local dendritic processing. (J. Rinzel and S. Baer)

Estimating the electrotonic structures of neurons. The usual estimation formulae (Rall, 1969) an equivalent cylinder model for the neuron. However, these assumptions are sometimes not valid, and even when these formulae are appropriate, some of the parameters may be difficult to estimate accurately. To tackle these problems, we have developed formulae for simple non-cylinder geometries. and these formulae minimize use of parameters which are difficult to estimate accurately. Also, for complicated morphologies, we have developed a computational approach. With a "forward computation", for a given morphology, we specify electrotonic parameters and evaluate quantities to compare with experiments. Alternatively, with an "inverse computation" we specify the parameters obtained from experiment and obtain the electrotonic parameters. (Holmes and Rall)

Long-term potentiation (LTP). Repetitive high frequency afferent stimulation may result in a large, enduring change (LTP) in the size

of post-synaptic potentials generated in a cell. Calcium influx through NMDA-receptor channels appears necessary for the induction of LTP. We have modeled calcium influx and control of NMDA-receptor channels in a hippocampal dentate granule cell taking into account detailed cell morphology and known biophysical parameters. For a sufficient number of co-active inputs, increasing input frequency results in a steep non-linear transition from low Ca^{++} to high Ca^{++} influx. Currently we seek to match the best temporal input patterns for LTP found experimentally with the model. (W. Holmes and W. B. Levy)

A Workshop on Reassessment of Dendritic Neuron Models was held August, 1987 at the Neuroscience Institute (Rockefeller Institute). The workshop generated agreement on a number of important issues related to fitting more complex theoretical models to the much more comprehensive anatomical and electrophysiological data that has recently become available, especially for spinal motoneurons. A three month visiting fellowship (by W. Rall) at the Australian National University in Canberra was devoted primarily to writing a joint manuscript to report the results of the workshop. (Rall, Burke, Holmes, Jack, Redman and Segev).

Computational models of small neural networks. This project involves development, analysis, and efficient implementation of numerical algorithms to solve the dynamical (e.g., cable) equations, and the graphical visualization and biophysical analysis of the results. For numerical integration of the Hodgkin-Huxley (HH) cable equations, we have used both the Crank-Nicolson method and the backward Euler method. The latter has been shown rigorously to converge for this nonlinear system. The method also forms the basis for a mathematical proof of the well-posedness of the initial-boundary value problem for the HH equations. These algorithms were implemented in a supercomputing environment, first to study recurrent negative feedback of a single neuron onto itself. We found behavior similar to that of high gain nonlinear amplifiers. Recently, a code has been written for the NCI Cray X/MP in Frederick which simulates the activity of an ensemble of HH-like neurons. These neuron models have both electrically active and passive regions (axons and equivalent cylinder dendrites) and may be arbitrarily connected into networks through model synapses. The code exploits vector processing on the Cray to achieve near optimal speed. Sizable amounts of data are generated. Dynamic visualization on the Silicon Graphics 4D/60T has been developed and sophisticated videotape for a model computation involving 64 neurons was produced. These techniques are now being applied (with W. Rall and J. Rinzel) to study a simple model of two synergistic motoneuron pools. (M. V. Mascagni)

Synaptic integration in the retina. Neurophysiological studies seek to understand the function of the first synapses of the visual pathway, and of the neural subcircuits where rods, cones, horizontal cells and bipolar cells interact. Previously, Nelson and Pflug found that dim backgrounds enhance the small-spot flicker-responses of cat retinal horizontal cells (type A) by a factor of 2 or more. Now they are measuring the spatial extent of this flicker-enhancement effect. Flicker enhancement is maximal with small squares or slits, but declines with stimuli beyond 500 microns width. A theoretical model, which involves the solution of two dimensional analogs of the cable equation, (S. Baer, R. Nelson and R. Pflug)

Electrical Oscillations in Nerve and Secretory Cells

Bursting electrical activity of insulin secreting cells. We continue to model the effects of coupling on pancreatic B-cells. Our new model for a single cell has a large number of high conductance, rarely open calcium-activated potassium channels. Because of this high conductance, a single opening can have a strong random perturbing effect. Thus our stochastic single cell model is consistent with experiments and exhibits irregular spiking, rather than bursting. We use this model to treat clusters of such cells which are so tightly coupled electrically that they effectively share a common pool of channels and a common membrane. As the number of cells in a cluster increases the spiking pattern becomes organized into bursting; the statistical fluctuations are smoothed because channel events are shared by an increasingly larger membrane area. Approximately 50 cells are required to attain regular bursting, in reasonable agreement with the limited experimental data. Although the treatment of coupling is over-simplified, the model supports the hypothesis that the emergence of bursting is due to channel sharing. For the next stage of modeling we view the islet as a collection of individually oscillating small tight clusters or cells, loosely coupled by gap junctions or diffusion of intercellular ions (K^+ and Ca^{++}). (J. Rinzel, A. Sherman and J. Keizer)

We continue to apply our simpler, deterministic model for representative cells in synchronized islets of Langerhans to analyze and interpret experimental data. Recently, A. Sherman (with Drs. Carroll and Atwater, LCBG, NIDDK) has considered the effects of pH on the glucose dose response of B-cells. Replacement of an HCO_3^-/CO_2 buffer by a HEPES buffer (which alkalinizes the cells) broadens the dose response curve and shifts the threshold to the right. This effect can be reversed by acidification. Modeling showed that this effect could be accounted for by blockage of a background voltage and calcium independent potassium current, presumably due to the ATP-sensitive channel. (A. Sherman, P. Carroll, and I. Atwater)

Phase plane analysis of neuronal excitability and oscillations. For an audience of neural modelers, we have illustrated the concepts of phase plane analysis and bifurcation theory by a thorough treatment of

the two-variable, Morris-Lecar model. For example, we contrast threshold behavior for different parameters: when the steady state current-voltage relation is N-shaped or monotonic. In the former case (Class I), threshold for action potentials is distinct, latency may be arbitrarily long, and intermediate-sized responses are not possible. In the latter case (Class II), action potential size may be graded, although generally quite steeply with stimulus strength, and latency is finite. Correspondingly, for a steady stimulus, the N-shaped case leads to onset of oscillations with zero minimal frequency (homoclinic bifurcation); in the monotone case it is non-zero (Hopf bifurcation). This project motivated the development (by G. B. Ermentrout) of a sophisticated interactive computer code (IBM PC-compatibles) for numerical and graphical analysis of arbitrary dynamical models. (J. Rinzel and G. B. Ermentrout)

The effect on threshold of a ramped stimulus. We have extended our study of excitable systems when a stimulus, or parameter, is slowly ramped through the threshold value for oscillations. Previously, we found that the stimulus value S_j at onset of repetitive activity is greater than the statically predicted threshold (the "delay" effect) and that S_j shows a curious, non-monotonic dependence on ramp speed. We demonstrated that the decrease in S_j for very slow ramps is due to the accumulation of fluctuations which diminish the delay effect. For the Hodgkin-Huxley model this decrease had previously been merely an observed phenomenon ("reverse accommodation"); our results provide a rationale for understanding it. We have demonstrated the delay effect in other Class II (see above) excitable systems, e.g. cAMP-receptor dynamics in *Dictyostelium discoideum*. In contrast, the Morris-Lecar (Class I) model does not show a delay. (Rinzel and S. Baer)

Reflection of impulses from axonal branch points and inhomogeneities. There is theoretical and experimental evidence for block for block of nerve impulse propagation at axonal branch points and other regions of low safety factor. For non-blocking (but nearby) parameter conditions, a "reflected" or "echo" wave may propagate back toward the stimulus in some models (e.g., numerical simulations of Goldstein and Rall). To identify the essential factors, we have formulated a simple two-cell model in which one cell presents a large capacitance-conductance load. To model excitability, we used both the Morris-Lecar and FitzHugh-Nagumo models as representatives of Class I and Class II systems (see above). We have seen echos only for the former case. It appears that long latency for action potential generation (due to a saddle point threshold) may play a key role for Class I axons. (J. Rinzel, G. B. Ermentrout and J. Bell)

We have extended our analytic study of relaxation oscillations characterized by two differing time-scales. Previously, we developed singular perturbation methods to study the transition to large amplitude oscillations as a control parameter was varied. For example, we estimated analytically the limit point for a subcritical bifurcation in the FitzHugh-Nagumo equation. We have now exploited a new singular limit that allows us to go beyond the limit point and further toward

the large amplitude regime. Although the analysis is local, the solutions capture "global" features, that until now, could only be obtained by numerical methods. (S. Baer and T. Erneux)

Cell Energetics

A diffusion-reaction model for ATP and its byproducts has been used to study concentration profiles in a renal cell during transitions in ion transport. The model consists of a spherical cell with mitochondria distributed in its interior and Na-K pumps at its periphery. Chemical reactions include oxidative phosphorylation at the mitochondria and dephosphorylation in cytosol and at the plasma membrane. Simulations using experimentally derived parameters indicate that neither ATP nor ADP are diffusion limited between the plasma membrane and cytoplasm. We considered the effects of various spatial distributions for ATP production, and decreased diffusivity (compared to that in water). (Lynch, Mejia and Balaban)

Renal Physiology

There are controversies in the theory of acid-base balance concerning mechanisms of pH regulation. We continue to develop a rigorous description of pH balance that is based on physical principles. Our canonical tube model consists of equations in space and time for solute, flow and charge conservation and Henderson-Hasselbalch equations describing chemical buffers. We have simulated experiments on the isolated, perfused rabbit cortical collecting duct. We conclude, for example, that carbonic anhydrase is not endogenously present in the perfused system. (Mejia and Knepper)

Previously, with J. L. Stephenson, we developed differential equation models that describe solute and water composition in nephro-vascular populations of the mammalian kidney. We have now incorporated more detailed anatomical data and physiologic parameters for water and solute permeabilities and other transport coefficients. We use CONKUB, our algorithm for continuing solutions of large systems of nonlinear equations, to obtain both steady-state and transient solutions to this model. Using parameters for the rat kidney, discretized equations for 2000 unknowns are solved interactively on a CRAY X-MP supercomputer, and transitions from one physiologic state to another are obtained in essentially real-time. (Mejia)

Atrial natriuretic factor (ANF) is a potent natriuretic and diuretic peptide which is secreted from the cardiac atria. The renal sites of action of ANF, and the mechanism by which ANF induces a hypernatremic natriuresis and diuresis are not currently known. We have used the central core model of whole kidney function (see above) to evaluate the effects on urine formation of three experimentally determined ANF effects in the kidney, specifically: increases in glomerular filtration rate (GFR), inhibition of active NaCl absorption in the collecting duct, and inhibition of osmotic water permeability in the collecting duct. The simulations show that all three effects cause an

increase in the excretion of NaCl and water, with inhibition of active NaCl transport causing the largest increase. While the model does not exclude any of the three experimental effects, it shows that only increased GFR delivers significantly more NaCl to the cortical collecting duct via the superficial nephrons. This suggests that micropuncture of distal cortical tubules of superficial nephrons will permit distinction between possible tubular and glomerular effects of ANF. (Mejia, Knepper, Sands)

Microcirculation and Facilitated Transport

The facilitated transport of oxygen mediated by myoglobin was studied in the presence of membranes, impermeable to the carrier, on the transport path. Some physicochemical entities are defined: the partition of flows between membranes, the flow transfers in the vicinities of the membranes, and the jump discontinuities of the oxymyoglobin concentration at the membranes. Analytic approximations for these entities and their relationships have been obtained. In our formulation, the flow transfer resistance involves an integral in some vicinity of a membrane; it is shown that this has advantages over the method of matching pointwise values of functions. We also reveal explicitly that three levels of description are involved: homogeneous neighborhoods for the diffusion reaction equation, spatial inhomogeneities introduced by the membranes with associated eigenvalues, and a global relation that governs the distribution of oxygen concentration at the membranes. The dependence of the facilitated transport on the parameters was analyzed. As the oxygen diffusion coefficient increases, the flow partition and the flow transfer resistance decrease so that the facilitated transport increases. As the carrier diffusion coefficient increases, the flow partition increases but the increase in the maximum possible facilitated transport overrides its effect, and the facilitated transport increases. Also, increasing either the "on" or the "off" chemical kinetics coefficient may increase or decrease the facilitated transport but through a different mechanism, their influences depend on the parameter values. (J. M. Gonzalez-Fernandez)

Auditory Physiology

The processing of complex sounds is examined theoretically and in model simulations at all stages of the auditory system. At the periphery, detailed cochlear models have been developed to account for the mechanics of basilar membrane motion and the biophysics of hair-cell function. These models are now regularly used to generate the cochlear responses to various speech phonemes and words. They form the front-end analysis stage in investigating more central auditory processing such as binaural hearing and sound pattern recognition.

Recently, we have developed and implemented detailed models of binaural processing that rely on spatial, rather than temporal, correlations to perform their function. Current efforts are focused on the adaptive mechanisms that can account for the formation of such networks during development. For the central processing and recognition of speech, adaptive algorithms have been developed that can encode and generate phonemic sequences. These algorithms utilize nonlinear phenomena such as hysteresis to encode the temporal information in a sequence, and thus offer an alternative to the biologically implausible wide use of multiple time constants in neural networks. Finally, intensive efforts are underway to combine these theoretical models with experimental data from ferret auditory cortex. Several studies dealing with various aspects of the collection and analysis of data have been completed this year. (S. A. Shamma)

Molecular Biology

Alignment of two sequences. Our previous methods have been improved so that identification of initial subsequence alignments (before complete optimization) now allow for insertions and deletions (and mismatches, as previously) with virtually no increase in computation time. The new method is included in our nucleic acid and protein search programs, and in a tool for evaluating statistical significance of similarities. Also, we have extended this approach for the analysis of local similarities between longer amino acid or DNA sequences. The enhancement gives a high resolution view of the subsequence and its flanks. The sensitivity and selectivity of the method is easily controlled by the user and the output may be as a standard alignment or in graphic matrix form. (D. Lipman and W. Pearson, U. Va. School of Med.)

Multiple sequence alignment. A recent algorithm (Carrillo and Lipman) renders feasible the optimal simultaneous alignment of as many as six sequences. Their algorithm, however, assumes the cost of a multiple alignment is the sum of the costs of its pairwise projections. (We call such alignments SP-alignments.) The most biologically realistic approach to multiple sequence alignment involves the minimization of the branch lengths of an evolutionary tree whose leaves are the sequences of interest. We have generalized Carrillo and Lipman's algorithm so that it can be used with tree-alignments. But, while tree-alignments are better justified than SP-alignments, algorithms for finding SP-alignments are more efficient and may therefore handle more sequences. We have developed several methods which use information from an evolutionary tree to derive weights for the pairwise distances of an SP measure. The goal is to obtain biologically realistic alignments with this more efficient measure. We have developed methods to compute these weights and are now evaluating the resulting alignments. We expect the weighted SP measure to be useful in a variety of other

sequence comparison problems. We are also developing software (with E. Myers & J. Kecicioglu, U. Arizona) which implements these tools in order to align up to seven amino acid sequences. Given a set of sequences, we compute an evolutionary tree based on the pairwise distances (which is output for the user), derive weights for the SP measure, and use a bounded shortest path approach to find an optimal alignment within an N-dimensional lattice. A prototype is now complete and is being evaluated. A new, rapid method for multiple sequence alignment, the Maximal Pairwise Alignment, is also being evaluated and may be included in the software package. (D. J. Lipman and S. Altschul)

A simply stated problem in matrix algebra arises from a problem in multiple sequence alignment (see above). While this problem can be solved by matrix inversion, its special structure permits a computationally more efficient solution by means of graph theory. Certain interesting graph theoretical constructs arise from the consideration. (S. Altschul)

Matrices for protein sequence comparison. Different matrices have varying success in distinguishing related sequences from those whose similarity is due to chance. We have developed a rationale for the construction of protein comparison matrices and are in the process of compiling statistics from related sequences that should help in the construction of more sensitive matrices. (D. J. Lipman and S. Altschul)

Identification of "motifs". We are developing flexible pattern matching software for evaluating sequence models ("motifs"), and for data base searches. Our method allows the user to define very general sequence patterns, and indicate the degree of confidence, or stringency, of each component of the model. Thus, the pattern may accept a variety of residues at a given site, as well as a variable number of gaps between specified sites. In addition, some sites may be more "valuable" than others and thus contribute more to a total match score. While attention has been paid to the flexibility of the patterns, and ease of use, we are also developing efficient algorithms for detecting matches. (D. J. Lipman and E. Myers, U. Arizona)

Recent data on enzyme electrophoretic mobility and DNA sequences for pertussis toxin allow the construction of evolutionary trees for various strains in the genus Bordetella. In contrast to previous analyses, these data can be seen to support the separate clustering of B. pertussis strains, in agreement with the traditional classification based on other phenotypic characteristics. An earlier argument placing the divergence of B. pertussis and B. parapertussis before 1912 does not follow from the newly proposed evolutionary trees, which also have different implications for several other claims concerning the evolution of these strains. (S. Altschul)

We have described an algorithm for finding the most significant repeating pattern in a single protein or nucleic acid sequence and used it to find an internal repeat in the messenger RNA for murine alpha-fetoprotein. (S. Altschul with J. F. Hasson and B. W. Erickson, U.N.C.)

NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 DK 13,001-15 MRB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mathematical formulations and analysis relevant to experimental neurophysiology.

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

PI: W. Rall Senior Research Physicist MRB, NIDDK

Others: W. R. Holmes NRSA Fellow MRB, NIDDK
I. Segev Visiting Fellow MRB, NIDDK

COOPERATING UNITS (if any) Dept/Neuroscience, Hebrew Univ. of Jerusalem

Lab. of Neural Control, NINCDS

Dept/Neurosurgery, U/VA Sch/Medicine

Physiology Lab, Oxford Univ.

J. Curtin Sch/Med., Australian Nat. Univ.

LAB/BRANCH

Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.8

PROFESSIONAL:

2.5

OTHER:

.3

CHECK APPROPRIATE BOX(ES)

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

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

RESEARCH AREA. Basic neuroscience involving structure/function relations for neuronal dendritic branching, dendritic spines, and synapses (also neuron populations with cortical symmetry), and for such functions as synaptic transmission, amplification and dendro-dendritic interactions in the context of spatio-temporal input patterns, logical processing of input, and neural plasticity, as in conditioning and learning.

RATIONALE. Combine experimental data from neuroanatomy and from electrophysiology with biophysical models of nerve membrane (passive, synaptic and excitable) into a comprehensive theory which can lead to new insights and to testable theoretical predictions (leading to the design of better experiments). To do this we must create explore and test mathematical and computational models with different degrees of complexity.

METHODOLOGY. Our methods include both analytical solutions and computational solutions of boundary value problems (for partial differential equations) in the tradition of classical physics. They include also the formulation and solution of problems in terms of systems of ordinary differential equations; when this is done explicitly for a compartmental model of a neuron, it is possible to accommodate a remarkable variety of dendritic branching patterns and non-uniform distributions of membrane properties and of synaptic inputs.

RESULTS. Earlier results are summarized in Chapt. 3 of "The Handbook of Physiology: The Nervous System, Vol. 1" published by the American Physiological Society, 1977 (Kandel, Brookhart & Mountcastle, eds.). More recent results are described in Chapter 22 of "Synaptic Function", Wiley, 1987 (Edelman, Gall & Cowan, eds.).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,002-16 MRB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mathematical description of substrate transport in capillary-tissue structures.

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

PI: J. M. Gonzalez-Fernandez Research Mathematician MRB, NIDDK

COOPERATING UNITS (if any)

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Mathematical Research Branch

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.03

PROFESSIONAL:

1.0

OTHER:

.03

CHECK APPROPRIATE BOX(ES)

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

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

The goal of this work is to develop mathematical models of the blood flow and transcapillary exchanges in capillary networks. An effort is being made to incorporate in the models the histological structure of capillary networks as well as different flow patterns from available experimental information. In this model the extraction of substrates with different chemical kinetics at the tissue site will be described. It is expected that this could be used in experimental situations where the extraction of different substrates are measured simultaneously, thus helping to infer the flow pattern features of the microcirculation. In particular a model of the diffusion-consumption of oxygen in striated muscle containing myoglobin (facilitated diffusion) is being developed and pertinent numerical results examined.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,004-14 MRB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mathematical description of cellular neuroelectric signal transmission.

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

PI:	J. Rinzel	Chief, MRB	MRB, NIDDK
Other:	S. M. Baer	Staff Fellow	MRB, NIDDK
	A. S. Sherman	NRC Fellow	MRB, NIDDK
	M. V. Mascagni	NRC Fellow	MRB, NIDDK

COOPERATING UNITS (if any) Dept/Mathematics, Univ. of Pittsburgh

Lab/Cell Biology & Genetics, NIDDK

Dept/Mathematics, SUNY, Buffalo

Dept/Chemistry, Univ/California, Davis

Dept/Neuroanatomy, Yale Univ.

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NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.9

PROFESSIONAL:

3.5

OTHER:

.4

CHECK APPROPRIATE BOX(ES)

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

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

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of neuroelectric signaling for individual neurons. Among the topics of current interest are: (i) integration of synaptic input delivered to the soma and dendritic branches of a neuron; (ii) propagation of action potentials along axons; (iii) stimulus-response and threshold properties for repetitive-firing of action potentials; (iv) complex bursting patterns of membrane potential oscillations which arise through endogenous membrane properties and/or interneuronal coupling.

Because qualitatively related mathematical or biophysical problems may arise in other contexts, e.g. chemical and biochemical oscillations, or e.g. excitation-secretion coupling, this project may consider models from such applications.

Mathematical models of these phenomena involve systems of linear and nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods drawn from both classical and modern applied mathematics. These methods may include finite difference or finite element numerical integration, bifurcation theory, perturbation techniques, and nonlinear dynamical systems theory. One goal of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet physiologically reasonable, equations.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,014-07 MRB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Probabilistic Analyses of Nucleic Acid Sequences.

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

PI: D. J. Lipman Research Scientist MRB, NIDDK

Others: S. F. Altschul IRTA Fellow MRB, NIDDK

COOPERATING UNITS (if any)

Dept/Computer Science, Univ. Arizona
Dept/Biochemistry, U/Virginia School of Medicine
Info. Tech. Br., NLM

LAB/BRANCH

Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.04

PROFESSIONAL:

2.0

OTHER:

.04

CHECK APPROPRIATE BOX(ES)

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

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

This project has focussed on the analysis of amino acid and DNA sequence data as it pertains to molecular biology and molecular evolution. Continuing areas of interest include:

The development of computational tools for molecular biologists. We developed a complete set of improved sequence comparison tools for molecular biologists which are being widely distributed. We have made an algorithmic breakthrough in the problem of multiple sequence alignment and are developing practical implementations. We are developing more-powerful and flexible pattern matching tools for use in data base searches and in the analysis of sequence/structure relationships.

We are working closely with the National Library of Medicine in their efforts to facilitate connections among the data in molecular biology data bases, to improve the useability of these data, and to provide a wide variety of supported, computational tools to the biologists on the NIH campus.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,017-05 MRB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Sound processing in the auditory system.

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

PI:	S. A. Shamma	Guest Worker	MRB, NIDDK
Others:	J. Rinzel	Chief, MRB	MRB, NIDDK
	R. Chadwick	Biomedical Engineer	BEI, DRS

COOPERATING UNITS (if any)

Biomedical Engineering & Instrumentation Br., DRS

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Mathematical Research Branch

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.4

PROFESSIONAL:

1.2

OTHER:

.2

CHECK APPROPRIATE BOX(ES)

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

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

The project involves research on the processing of speech and complex sounds at three levels of the auditory system:

1. Peripheral stages - theoretical models of cochlear function to explain the results of various physiological and psychophysical experiments.
2. Neural network models to process and extract important parameters of speech and other sounds for both monaural and binaural hearing.
3. Learning algorithms mimicking adaptive central auditory neural networks to perform storage and recognition tasks.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,019-04 MRB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mathematical study of excitability properties in coupled nerve membrane patches.

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

PI: S. M. Baer Staff Fellow MRB, NIDDK

COOPERATING UNITS (if any)

Dept. Engineering Sciences & Appl. Math., Northwestern Univ.
Dept. of Biological Sciences, Florida State U., Tallahassee

LAB/BRANCH

Mathematical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.60

PROFESSIONAL:

1.58

OTHER:

.02

CHECK APPROPRIATE BOX(ES)

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

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

Biophysical theory regards the electrophysiological interaction between passive and active patches of nerve membrane as functionally significant. Important examples include dendritic spines with both passive and excitable spine head membrane, myelination, and the interaction between dendrite and axonal membrane.

The aim of this project is to explore, using mathematical modeling, analysis, and numerical computation the functional implications of these interactions.

Last year we expanded our research efforts to include a new class of excitability problems involving a slowly-varying control parameter. We are pleased to report that this study revealed some new and valuable insights into the biophysical phenomena of accommodation.

Areas of research initiated this year include the evaluation and study of the dynamics of an alternative mathematical model of the Hodgkin-Huxley squid data and applications of cable theory to assist experimenters in understanding the function of the first synapses in the visual system.

ANNUAL REPORT OF THE LABORATORY OF CELLULAR AND DEVELOPMENTAL BIOLOGY

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

A broad spectrum of types of research fall under the rubrik of cellular and developmental biology as studied by the forty scientists and ten support personnel of LCDB. At one end of this spectrum are atomic and molecular resolution studies of proteins and protein-DNA complexes; at the other are studies of mammalian development and genetic defects. Between these are groups interested in genetics and gene regulation in yeast, *D. discoideum*, sea urchins and human tissue culture cells; ultrastructure; biotechnology; hormonal regulation mechanisms and oncogenes. Most of the groups share common technological approaches in their work; this, together with an environment which fosters communication, leads to highly productive interactions among all groups within the laboratory, in spite of the seeming diversity of research areas under investigation.

The past year has seen significant progress in all the areas studied in LCDB. In addition to research papers, members of the laboratory have published several critical invited reviews and presented data at universities, other NIH laboratories, and national and international meetings. Of particular note this year was publication of a volume of *Methods in Enzymology* on molecular cloning technology which was co-edited by Alan Kimmel. The book is a major compendium of cutting-edge techniques in recombinant DNA work and was reviewed enthusiastically by *Nature* recently.

After several years of illness, Dr. Herbert Windmeuller retired this year. Herb was attracted to NIH by Dr. John Bieri and was an active member of the Nutrition Section of the Laboratory of Nutrition and Endocrinology (our progenitor) for many years. He is recognized as an expert on the metabolism of the small intestine; his studies on glutamine metabolism in this organ and liver are often-cited classical investigations. More recently, he investigated the synthesis of apolipoproteins in liver and small intestine. His research on the effects of physiologic state on relative rates of synthesis of several variant forms of apolipoprotein B now takes on increased importance with the recent demonstration of a unique method the cell has developed to synthesize the different proteins from a common gene. The institute is planning a symposium this Fall to honor Herb Windmeuller and his contributions.

The following summary of research in LCDB for 1987-1988 is organized as we have done previously. Rather than summarize experimental work done by individual Sections or working groups, I choose to review progress in a thematic sense, by the types of research done in the laboratory. This approach emphasizes the continuity of the spectrum of research done in LCDB and will hopefully lead the reader to an appreciation of the interactions which make the laboratory more than the sum of its component parts.

Structural Studies

The use of barnase, an extracellular ribonuclease of *B. amyloliquefaciens*, and barstar, its cognate intracellular inhibitor, as a system for investigation of protein folding mechanisms and protein-protein interactions was markedly facilitated several years ago by cloning and overexpression of the barnase

gene, followed in a year by similar achievements for the barstar protein. Routinely, 100 mg quantities of either protein can be produced from a liter culture of *E. coli* bearing an appropriate genetically engineered plasmid. Efforts are now underway to characterize the structure of barstar and the 1:1 complex of the two proteins.

The barnase:barstar complex is stable to the conditions necessary for gel electrophoresis, providing a facile method for analysis of the effect of mutations on association properties of the proteins. An active site mutation (His-->Gln102) of barnase abolishes catalytic function without affecting protein folding or interaction with barstar. Alteration of either of the two cysteinyl residues (40 or 82) of barstar to serine does not grossly affect the inhibitor's interactions with barnase. A new approach to defining residues that may be of little importance in protein folding and interactions for this system involves looking for natural polymorphisms in several independently isolated strains of *B. amyloliquefaciens*. Ruling out particular residues as being of importance reduces the work load for creation of mutants. Initial screening using RFLP's allowed separation of the strains into four groups. Cloning and sequencing genes from a representative of each group has shown fewer than four conservative amino acid differences between barnases and even fewer between barstars.

A collaborative effort has been established with several European laboratories to advance study of the barnase:barstar system. Early results suggest that nuclear magnetic resonance techniques may prove capable of solving the structure of the inhibitor protein. Given the advances in multidimensional NMR that are forthcoming and the known X-ray structure of barnase at high resolution, knowledge of the barstar structure may allow solving of the solution structure of the complex in the future by NMR.

Gene regulation in eukaryotic cells occurs in the context of chromatin, a complex of DNA, histones, and nonhistone proteins; this makes understanding of chromatin structure a prerequisite to the understanding of transcriptional regulation. Five years ago, we described a DNA segment which had sequence information that led to formation of a precisely positioned nucleosome core particle when the cloned DNA fragment was associated with histones. DNA constructions based on that sequence have been made and are being used in collaborative structural studies with several laboratories. A 146 bp length has been overproduced in 50-100 mg quantities, associated with the histone octamer and crystallized. These core particle crystals are being used as subject for the first high resolution X-ray analysis of core particle structure by Dr. Tim Richmond at the ETH/Zurich. The crystallographic information obtained in the first diffraction patterns indicates that nearly three fold more data can be obtained from the unique sequence core particle than was available using a random sequence core particle crystal preparation. Tandem repeats of the DNA sequence, engineered to give different repeat lengths and different numbers of repeat elements were made as a model for the study of higher order chromatin structure. These DNA molecules have been distributed to a number of laboratories (van Holde, Bradbury, Langmore, Widom, Gould, Isenberg, etc.) where they are being actively studied by a variety of biophysical techniques.

Previous reports from this laboratory have documented the occurrence and, in several cases, the mechanism for sequence specific association of DNA with

histone octamers, leading to what have been called positioned nucleosomes. An intriguing thought deriving from studies of positioned nucleosomes has been the possibility that positioning may be involved in the functional features of DNA sequence motifs. Several DNA segments, including promoters and replication origins, are found in chromatin as the terminal region of a nucleosome core particle. Many years ago, we described the dynamic nature of the terminal 20 bp at each end of a core particle. We now ask whether nucleosome positioning serves a functional role. Our first attempt to address this question uses the core consensus ARS sequence in the TRP1ARS1 plasmid. The experiment involves creating mutant ARS's which will shift the consensus core DNA into a nucleosome by 5, 10, 15 or 20 bp. These mutant ARS's are cloned in plasmids which also contain an ARS sequence adjacent to the GAL1/10 UAS. Based on experiments of others, we surmised that ARS function would be abrogated by transcription through the region when the GAL locus is activated by growth of cells in galactose, as is centromere function. This has proven true, both in our experiments and in recently published work of others. The DNA constructs have been made and current experiments are providing the beginning data in determining whether nucleosome positioning serves in regulation of chromatin replication. Extensions of these studies will address the related question regarding transcriptional initiation.

Chromatin Organization, Transcription and Regulatory Factors

Evidence suggesting that nucleosome positioning may be important in transcriptional regulation has been obtained in studies of the *Xenopus* 5S rRNA gene. DNA containing the gene, or variants thereof, was associated with histones in vitro and transcription evaluated on incubation in egg extracts. Association of plasmid DNA with increasing amounts of chicken erythrocyte histones led to lessened levels of transcription, indicating that histones interfered with transcription initiation, elongation, or both. To investigate the effect of specific nucleosome localization on in vitro transcription in more detail, we devised an approach which uses restriction endonucleases to select subpopulations of reconstituted minichromosomes which have nucleosomes located at specific sites, and then evaluated transcription of these molecules. We have found that nucleosomes on or in the vicinity of the TFIID binding domain completely inhibit transcription. To evaluate the effect of nucleosomes on elongation, we have constructed extended genes with elongated transcription regions downstream of the TFIID binding site. Data using these constructs suggest that RNA polymerase III can transcribe part way into a nucleosome which includes the termination signal, but cannot transcribe completely through a nucleosome. These data suggest that nucleosome disruption may be necessary for transcription of segments of chromatin longer than one nucleosome. We have examined the topological properties of chromatin DNA before and after transcription of the HSP26 gene in yeast. Our data do not provide any indication that nucleosome disruption lasting longer than five minutes (the lower time limit for resolution of topological changes) occurs. If both suggestions are true, then alteration of nucleosome structure concomitant with transcription must be transient and rapid.

Physical studies of DNA topologic constraints in plasmid chromatin support the idea that there may be fundamental structural differences between transcriptionally active and repressed chromatin. Previously, we had shown that chicken erythrocyte histones constrained thermal untwisting of about 200 bp of DNA per nucleosome. More recently, we, and others, showed that yeast histones only

constrained about 30% of their associated DNA. Using a matrix composed of various DNA sequences, assembled with histones from different sources, either in vivo or in vitro, and relaxed with different topoisomerases, we have now clearly shown that differences in the constraints enforced upon DNA between yeast and chicken erythrocyte must arise from differences in the two histone populations. Since the genome of yeast is about 50% transcriptionally active while that of larger eukaryotes is about 3-5% active, these differences may reflect a more flexible chromatin structure for transcribed regions of the genome.

We have previously detailed results of study of the structure of yeast TRP1-ARS1 plasmid chromatin. This 1453 bp episomal element contains two nuclease hypersensitive regions, one near the 5' end of the TRP1 gene and one near the 3' end of the gene and the functional ARS (replication origin) sequences. There are four unstable nucleosomes on the TRP1 gene. On a region of unknown function (UNF) between the ARS and the 5' end of the gene are three stable, precisely positioned nucleosomes. We have developed methods to purify the minichromosome using, initially, conventional biochemical methods, and more recently, protein-nucleic acid affinity. We have inserted several regulated yeast genes into the plasmid DNA, transformed yeast with these constructs, and now compare the composition and structure of the minichromosome containing the regulated gene when the gene is repressed with those features of the actively transcribed gene.

Because the TRP1ARS1 plasmid replicates to about 100 copies per cell, it is necessary to demonstrate, prior to attempting a full scale purification, that regulation of an amplified gene is similar to that for a unique, genomic copy. Last year we reported RNA abundance studies which indicated that the heat shock gene HSP26 is regulated normally when present on the TRP1ARS1 plasmid. We have now found that genomic and plasmid borne HSP26 genes also are virtually indistinguishable in terms of chromatin structure. Nuclei from parent and plasmid containing cells were digested with nucleases and cutting sites mapped using indirect end labeling. The gene itself appears to be packaged in five nucleosomes while the region 5' to the transcription unit has three nuclease hypersensitive regions; each region consists of a pair of hypersensitive sites separated by about 50 bp. These structural features are present on both the genomic and the plasmid HSP26 chromatin.

Chromatin structure of the HSP26 gene was nearly identical before and after induction of transcription, in contrast to changes in the nuclease sensitivity pattern of heat shock genes in *Drosophila* upon heat shock. This suggests that the heat shock regulatory protein (HSF) in yeast may be constitutively bound to its target site on DNA. To examine this further, we adapted a gel mobility shift assay to detect HSF bound to HSP26 chromatin. Plasmid chromatin was purified to a point where no free or nonspecifically bound HSF could be detected. Presumptive HSF was found in the proteins isolated from plasmid chromatin in both control and heat shocked cells, supporting the contention that HSF is constitutively bound. This finding requires reinterpretation of current ideas of how HSF might regulate transcription of these genes. It is also the most direct demonstration thusfar supporting the use of plasmid chromatin isolation as a method for identification of trans-acting factors and characterization of the structural role they play in control of transcription.

We have also studied the chromatin organization of the yeast 5S rRNA gene inserted and amplified in the TRPLARS1 plasmid. While the gene is not regulated, the 5S gene is one of the best studied transcriptional entities in biology; nevertheless, elucidation of the interplay between transcription factors IIIA, IIIB, IIIC and RNA polymerase III has not been possible. Indirect end label analyses were used to address the structure of the 5S gene in plasmid chromatin. A nuclease hypersensitive site is present within the 5S gene (this gene has a known intragenic promoter) and another site is located in the 5'-flanking region. Primer extension footprinting is currently being employed to correlate the hypersensitive sites with specific protein-DNA interactions in the putative transcription complex.

These studies, aimed at elucidating the structure of complexes containing previously identified trans-acting regulatory factors, provide a sound base for other investigations which have as their goal the identification of such trans-acting factors in more complex systems. One of these under development for the past two years is the search for proteins involved in the tissue and developmental stage specific regulation of human globin genes. The gene of particular interest is the epsilon-globin gene, a member of the beta-globin cluster expressed during embryonic life. K562 cells, derived from a human leukemia, express this gene. Nuclease hypersensitive sites are present in both 5' and 3' flanking regions when the gene is expressed; the 5' region contains a number of important sequence elements common to either most eukaryotic genes or to all globin genes and the 3' region contains sequences similar to described enhancer element sequences in human A-gamma- and beta-globin and chicken beta-globin genes.

Using mobility shift assays and footprinting, we have shown the presence of several proteins which appear to bind in a highly specific fashion to regions in the 200 bp to the 5' side of the transcription initiation site. However, similar patterns were observed for uninduced and induced K562 cells and for nuclear protein extracts from non-erythroid cells, suggesting that the interactions reflect factors that are not involved in the specific activation of globin transcription. In contrast, two highly specific complexes were formed with uninduced or induced K562 extracts and an oligonucleotide derived from the 3' flanking hypersensitive region; these complexes were not found in extracts from non-erythroid cells. These erythroid specific complexes also formed with sequences from the human A-gamma- and beta-globin enhancers. Further characterization of these factors and their interactions with controlling DNA regions should help in elucidation of the mechanism of developmentally specific gene regulation of the human globin genes.

Developmentally Regulated Genes

Another set of mammalian developmentally regulated genes of interest are those coding for the three proteins of the murine zona pellucida, an extracellular glycocalyx which surrounds the growing oocyte, functions to mediate species specific sperm interactions, prevents polyspermy, and protects the embryo prior to blastocyst implantation. We have reported previously cloning of cDNA and genomic clones for ZP3, the sperm receptor protein, characterization of the timing of expression of the gene, and its chromosomal localization.

We have now determined the genomic organization of the ZP3 gene. The gene has eight exons, ranging from 92 to 338 bp, flanked by consensus splice donor and

acceptor sequences. Introns of length 195 to 2520 bp lead to a span of about 8.6 kbp for the entire gene. S1 nuclease analyses of the 5' and 3' ends of the transcript defined relatively short untranslated regions of 29 and 16 nucleotides, respectively.

To help in defining regulatory elements involved in ZP3 gene expression, we have sequenced about 2 kbp of flanking sequences. Several elements often found in 5' flanking sequences of eukaryotic genes are present: TATA box at -29, an SP1 binding site homolog at -45 and the conserved half of the recognition sequence of CTF/NF1 at -240. Further upstream, -507 to -826, are six copies of an imperfect (but 88% conserved) 54 bp repeat. A similar repeat reiterated five times is found in the seventh intron. The mouse genome contains about 1000 copies of the repeated element, which lacks significant similarity to any sequence in the GenBank data base.

At the other end of the gene, sequences thought to be important for formation of 3' termini are found 8 and 181 bp past the poly(A) addition site. Forty three bp from the 3' end of the gene is a tandem array consisting of 12 nucleotides perfectly repeated 11 times. This element will have a distinctive secondary structure; its role in gene activity is a matter for speculation.

Completion of the genomic ZP3 sequence allowed determination of the missing 46 bp at the 5' end of ZP3 mRNA that were not represented in the cDNA clones. An open reading frame of 1272 nt begins in the usual vertebrate ANNATG motif. It seems likely that a signal peptide of 22 amino acids is present in the protein; cleavage between SER22 and GLN23 followed by cyclization of glutamine to pyroglutamic acid would be consistent with the inability to identify the N-terminal amino acid of secreted ZP3 by Edman degradation. Six potential N-linked glycosylation sites are present in the protein. Consistent with the postulated signal peptide role, the first 17 amino acids are quite hydrophobic. Strikingly, a 26 amino acid segment near the carboxyl terminus has a hydrophobicity index normally associated with transmembrane protein regions; its role in protein structure or protein-protein interactions remains to be determined.

We previously reported a ZP2 cDNA clone isolated using immunological methodology similar to that used for the ZP3 cDNA's. Rescreening a lambda library with the ZP2 cDNA has allowed isolation of overlapping clones which span the ZP2 message from an initiator ATG to the poly(A) tail. ZP2 has neither nucleic acid nor amino acid sequence similarity to ZP3. Genomic clones have been isolated using the ZP2 cDNA and characterization of these is currently underway.

Additional experiments in this project aim to enhance understanding of the biological activities of ZP3 protein. We are developing expertise in the culture, transfection and insertion into blastocysts of embryonic stem cells in order to establish transgenic mouse lines for study of the role of ZP3 in oogenesis and early development. We have also developed, in collaboration with members of Dr. Bernard Moss' laboratory, a CV-1 cell vaccinia virus based expression system for production of mutated forms of ZP3 as a glycoprotein. We will examine structure-function relationships for ZP3 with regard to induction of the acrosome reaction and mediation of species-specific sperm binding.

In a much smaller organism, yeast, we use genetics to study the mechanism of regulation of a particular gene under control of the MAT locus (mating type).

The products of the MAT locus are regulators of large batteries of genes which specify cell type. Several of the MAT locus proteins are DNA binding molecules which regulate transcription of target genes. We study a strain of yeast which secretes amylase under control of the MAT locus. The enzyme is secreted by haploid cells and by cells homozygous at MAT, but not by heterozygous MAT diploids. We constructed a complete deletion of MATA2, one of the two known transcriptional units of MATA and showed that this deletion does not affect MAT regulation of amylase secretion. A comprehensive analysis of the deletion demonstrated clearly the efficacy of the mutation. These results contradict those of other workers using only genetic methods, methods which are much less direct than the approach we have taken. Experiments currently underway will delete the other transcriptional unit at MATA and assess the effect on amylase secretion. We will determine the effects of overexpression of MAT proteins.

Mechanisms of Hormone Action

Signal transduction has long been an interest of members of LCDB who study the mechanisms whereby hormones regulate cellular metabolism, particularly in isolated adipocytes, a model for molecular endocrinology established by Dr. Martin Rodbell, a former member of the laboratory. Recently, improvements in the isolation of rat fat cells have led to a preparation which is highly reproducible in its metabolic characteristics and accurately mirrors the *in vivo* situation. Current interests involve the regulation of lipolysis by hormones which stimulate adenylate cyclase and by insulin. We use relative activation of A-kinase as a measure of intracellular cAMP concentration. In the past we have shown that insulin inhibits lipolysis in both a cAMP-dependent and a cAMP-independent fashion. Our interpretation of the data led us to suggest that insulin might lead to activation of a phosphatase which reversed post-translational modification of hormone sensitive lipase, leading to the cAMP-independent inhibition of lipolysis.

We have examined the phosphorylation state of a variety of cellular proteins under different physiological conditions. After loading cells with $^{32}\text{P}_i$, a 65 kD protein localized in the lipid fraction of adipocytes was examined under conditions where cAMP concentrations were held invariant. Kinetics and concentration dependence of the labeling suggested that concerted action of a kinase and a phosphatase lead to a steady-state level of phosphorylation. Activation of A-kinase leads to activation of a phosphatase that removes phosphate from A-kinase substrate proteins. Addition of insulin lead to removal of phosphate from the 65 kD protein and other A-kinase substrates. This concerted action is consistent with our previous interpretation of the possible mechanism of cAMP-independent inhibition of lipolysis by this hormone.

Another protein of interest is a 62 kD peptide; its phosphate content rapidly and dramatically increases when adipocytes are exposed to insulin. This phosphoprotein is also found exclusively in the lipid fraction of fat cells. Only threonine residues are modified in the protein, even though insulin action is known to lead to activation of a receptor-associated tyrosine kinase as an early event. Increases in A-kinase activity abolish insulin stimulation of phosphorylation of this substrate. Together, these data suggest a complex interplay or "crosstalk" between insulin and adenylate cyclase linked receptors, their transducers, or second messenger pathways.

Obviously, we would like to directly address the modification state of hormone sensitive lipase (HSL), as opposed to looking at the phosphorylation of these more abundant proteins even though the modification of the 65 kD protein follows that suspected for HSL. We have purified HSL and produced a polyclonal antiserum against the protein in rabbits. This antiserum was generated with SDS-PAGE isolated protein as antigen; it neither inhibits HSL activity nor immunoprecipitates the native enzyme. We have therefore used the antiserum to screen a fat cell cDNA expression library in hopes of isolating a clone that bears the epitope(s) recognized by the antiserum. Thusfar this approach has not succeeded. We then sequenced a peptide derived from purified HSL and have (i) synthesized an oligonucleotide corresponding to the peptide for use in screening the cDNA library and (ii) synthesized the peptide to use as antigen for producing antibodies to facilitate study of HSL phosphorylation in fat cells. We are confident that one of the approaches will provide us with the molecular reagents necessary for further investigations.

We have studied extensively the G proteins, constituents of the transducing pathway which also includes hormone receptors, proximally, and adenylate cyclase, distally. Data reported last year suggested that such GTP-binding proteins, especially G_i , were located on intracellular membranes, in addition to plasma membranes, and that these G proteins translocated in response to insulin as does the glucose transporter. More recent experiments have suggested a seasonal variation in the localization of these proteins. With the availability of antibodies specific for various subtypes of the G protein family, we have found that three members of the alpha G_i family, 1, 2, and 3 are present in adipocyte membranes along with a modest amount of alpha G_o . The fat cells contain a relatively large amount of G_{i3} alpha protein; this is the subspecies which is most susceptible to insulin-induced translocations between various membrane fractions. Heterogeneity in the G_i protein composition of adipocyte membranes may relate to the variations in translocations we reported previously.

Fat cells possess the typical complement of G_s proteins, as measured with specific antibodies. However, in addition to the 41 and 45 kD proteins in plasma membranes, we have found a new protein, 55 kD in size, in high density microsomal membranes. The protein was defined as a G_s family member by reacting with antibodies specific for the family and ADP-ribosylation by cholera toxin in intact cells or by cholera toxin and NAD in isolated membrane preparations. In vitro ADP-ribosylation of the 55 kD protein required addition of ARF, an ADP-ribosylating factor necessary for toxin action on other purified G proteins, suggesting that this molecule is a bona fide member of the G_s protein family. All these studies of signal transduction in the well studied adipocyte complement in bidirectional fashion the studies of paracrine hormonal regulation of differentiation in *D. discoideum*, for the benefit of both research areas.

An interest in signal transduction and mechanisms of differentiation that lead to hormonal responsiveness has generated a system under investigation in LCDB for several years. MDCK cells lose responsiveness to glucagon when transformed by Harvey MSV. A number of small molecules, most notably prostaglandins, lead to a return of hormone responsiveness when cells are cultured in their presence. Coordinated with the development of glucagon responsiveness is a decrease in the expression of p21, the product of the ras oncogene. A number of oncogene proteins are now known to be normal cellular constituents which

are often involved in either growth control or response to growth factors. The ras gene product has been shown (or suggested) by others to be involved in cAMP-mediated transduction, phosphoinositol signalling, and certain functions of EGF. We are now addressing the relationship of ras expression to signal transduction.

A transfected NIH 3T3 cell line containing a ras oncogene under control of the MMTV steroid-inducible promoter shows a decrease in EGF binding when transcription of the oncogene is induced by dexamethasone. Whether the loss of EGF binding is due to activation of protein kinase C by elevated concentrations of diacylglycerol in the ras overexpressing cells is under investigation. We have transfected MDCK cells with plasmids containing cellular and viral ras oncogenes under control of the glucocorticoid inducible promoter to extend these studies to the differentiated cells which normally express the glucagon receptor moiety of the signal transduction apparatus.

Lipid Metabolism

In addition to the direct study of mechanisms of hormone action on fat cells, others in the laboratory study genetic defects in lipid metabolism, enzymes involved in lipolysis and clinical manifestations of lipid disorders. Mice born with combined lipase deficiency (cld/cld, a recessive mutation in the T/t complex of chromosome 17) develop extreme hyperchylomicronemia and die within three days if allowed to suckle. They have very low levels (<5% of normal) of lipoprotein and hepatic lipase activities, their tissues are virtually devoid of fat, and 95% are tailless. Studies in vivo showed that brown adipose and other tissues of the cld/cld mice synthesized normal sized lipoprotein lipase protein but the enzyme was inactive and not transferred to capillaries, the normal site of action of the enzyme.

We found that cells cultured from brown adipose tissue of 1-d old cld/cld and unaffected mice readily differentiated into brown adipocytes. Lipoprotein lipase activity in unaffected cells was low on day 2 of confluence but increased ten-fold by day 6. In contrast, lipase activity in cld/cld adipocytes was always less than 1% of normal. Unaffected brown adipocytes spontaneously released lipoprotein lipase to the medium and also released lipase activity in response to heparin. On the other hand, cld/cld adipocytes released no lipase activity even in the presence of heparin, although these cells contained twice as much immunoreactive lipase protein as unaffected cells. We conclude that cld/cld brown adipocytes synthesize an inactive, nonsecretable form of lipoprotein lipase.

Lipoprotein lipase was immunolocalized in cultured brown adipocytes. Localization of lipase in unaffected cells was difficult because of the rapid turnover of the protein. Lipoprotein lipase, a glycoprotein, was retained in these cells when treated with either tunicamycin, an inhibitor of N-glycosylation, in the endoplasmic reticulum, or monensin, an inhibitor of transport of glycoproteins from the Golgi apparatus to the cell surface. We had found earlier that lipoprotein lipase synthesized by 3T3-L1 adipocytes in the presence of tunicamycin was smaller on SDS-PAGE than that synthesized by untreated cells, reflecting inhibition of glycosylation.

Fluorescent immunolocalization of lipoprotein lipase in unaffected cells showed the lipase in a reticular pattern throughout the cell, representative

of endoplasmic reticulum, in cells treated with tunicamycin, and primarily in the Golgi of cells treated with monensin. Lipoprotein lipase in cld/cld adipocytes, however, was found only in the reticular pattern, regardless of how cells were treated. Electron microscopic localization confirmed that lipase was present only in the endoplasmic reticulum of cld/cld brown adipocytes. Studies in vivo showed that lipoprotein lipase synthesized in cld/cld tissues is normal in size. These findings suggest that lipase synthesized in cld/cld mice is glycosylated in endoplasmic reticulum, accepting a high mannose type oligosaccharide. However, the lipase is not transported to the Golgi where the oligosaccharide component would normally be modified so that the enzyme would be active and be secreted by the cell.

We also study lipid metabolism in human diseases such as type C Neimann-Pick (NP-C) disease, an autosomal recessive neurovisceral lipid storage disorder. Fibroblasts derived from patients with NP-C accumulate excessive amounts of unesterified cholesterol when incubated with LDL. We have used fluorescent probes to determine the sites of cholesterol accumulation. Filipin is a probe that forms fluorescent complexes with cholesterol and causes characteristic membrane alterations visible in the electron microscope. We have also used a monoclonal antibody to cholesterol and a monoclonal antibody to a lysosomal membrane component for localization of cholesterol. Abnormal cholesterol accumulation in NP-C cells occurs not only in lysosomes but also in the Golgi complex. These findings suggest that components of the Golgi complex may play a role in the intracellular translocation of exogenously derived cholesterol and that disruption of the cholesterol transport pathway at the Golgi could be responsible for the deficiency in cholesterol utilization in NP-C fibroblasts.

We have used an immunocytochemical assay to determine the location of unesterified cholesterol in a variety of cholesterol enriched membranous particles including suspensions of liposomes, low density lipoproteins (LDL), hydrolyzed LDL and aortic plaque particles. Control experiments showed that high concentrations of unesterified cholesterol were detected by the antibody. Immunolocalization of cholesterol has advantages over filipin treatment for the detection of cholesterol in that it does not distort membrane structure. Aortic plaque particles isolated from rabbit and human atherosclerotic lesions enriched in unesterified cholesterol were immunolabeled. The cholesterol antibody labeled an amorphous structure associated with the surface of membranous particles and bundles of collagen.

Fatty acids and monoacylglycerol produced during absorption of dietary fat are generally thought to be solubilized and transported in intestinal contents as micelles formed with bile salts. Recent findings reported elsewhere indicated that lamellar structures coexist with mixed micelles and could be involved in transport of lipolytic products into intestinal cells. We investigated this possibility by study of the effects of various substances on activity of pancreatic lipase and release of lipolytic products to aqueous media. Droplets of trioleoylglycerol suspended from the top of a flow through chamber were perfused with lipase and then with lipase-free media containing different additives. Changes in pH and ionic strength of the perfusate, as well as addition of bile salts and/or calcium affected the rate and extent of lipid hydrolysis and the release of lipolytic products.

Morphological observations indicated that lipolytic products formed bilayered, lamellar structures, sometimes vesicular, before they were disrupted by bile

salts in the aqueous media. These observations confirm reports by others that liposomes may coexist with mixed micelles in intestinal contents during fat absorption, suggesting that lamellar structures could be involved in transport of lipolytic products into intestinal cells. These findings support Scow's earlier proposal that fatty acids and monoacylglycerol could be transported from dietary fat droplets into epithelial cells by lateral flow in an interfacial membrane continuum.

Biotechnology and Applied Biochemistry

Several areas of research in LCDB are closer to bringing the results of basic investigations to use in clinical situations or for production of materials for other research. I have grouped them together in this section of the report, even though their subject matter is not logically coherent.

The Biotechnology Unit is a service facility for all of NIH in addition to serving in research and development of biotechnological methodology, particularly production of microbial, fungal and tissue culture cells and initial large scale processing of biologicals. During the past year over 120 fermentations in volumes from 10 to 300 liters were performed. Mammalian tissue culture cells were grown for various groups at NIH in volumes up to 50 liters. A number of fermentations were for production of toxins for potential clinical applications; notable were growth of *Bordetella pertussis* for toxin purification and toxoid preparation to be used as a more efficacious whopping cough vaccine and growth of *Pseudomonas aeruginosa* for isolation of exotoxin to be used in development of antineoplastic directed cytotoxic agents. Development studies concentrated on improvement of yields of toxins and biomass.

Noted before were studies of ZP3, the murine sperm receptor. We had shown previously that antibody to ZP3 could be used to passively immunize mice against conception in an effective, but reversible, fashion. We have used several strategies to try to develop an active contraceptive vaccine. First, we cloned short segments of ZP3 cDNA into an expression vector, localized the clones that expressed the epitope recognized by a monoclonal antibody known to confer passive immunity against contraception, and determined the amino acid sequence of the epitope. This seven amino acid peptide was then synthesized, coupled to a carrier protein and used to immunize mice. Antibody to the complex was produced in female mice. Titers were sufficiently high that antibody could be found bound to the zona surrounding growing oocytes in the murine ovaries. Studies currently underway will determine if the antibody functions to prevent fertilization. Second, we are making a recombinant vaccinia virus which expresses ZP3 protein from a full length cDNA. Thusfar, we have made a shuttle vector which directs the synthesis of secreted ZP3 protein when transfected into CV-1 cells infected with vaccinia. This plasmid will be used to make the recombinant virus in order to vaccinate mice and test them for fertility.

Studies of dihydrofolate reductase, the target enzyme for methotrexate, an antimetabolite of high clinical importance in treatment of cancer and autoimmune diseases, have been carried out in LCDB for more than two decades. Collaborative investigations of (i) the structure and (ii) interactions with drug derivatives of the enzyme from several vertebrate sources continue. Another interest concerns the origin and significance of a lag seen in the kinetics of the sheep enzyme and absent for several other proteins. Increases

in ionic strength abolish the lag and significantly increase the enzymatic rate.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15004-13 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Modulation of Hormone Responsive Systems by Ras Oncogene Product

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

P.I.:	Michael C. Lin	Research Chemist	LCDB:NIDDK
Others:	Yvonne Wu	Senior Staff Fellow	LCDB:NIDDK
	Beatrix White	IRTA Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

Eugenio Santos, LLM:NIAID
K. P. Huang, ERRB:NICHD

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

3.2

PROFESSIONAL

2.4

OTHER:

0.8

CHECK APPROPRIATE BOX(ES)

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

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

The goal of our research is to understand the modulation of signal transduction mechanisms during the expression of a well-defined oncogene. Our previous finding that ras transformation of a kidney cell line (MDCK cells) leads to loss of glucocorticoid sensitivity, raises a possible role of ras product in hormone signal transduction. From our studies and others, it has been shown that ras transformation not only affects cyclic AMP-mediated signal, it also elevates phosphoinositol level and abolishes certain functions of EGF. In order to examine the causal relationship between the expression of ras gene and the various changes of signal transduction, a new approach has been taken to allow more precise control of the production of p21. We have obtained a transfected NIH-3T3 cell line containing MMTV LTR ras oncogene. Our preliminary results show that in the presence of dexamethasone in culture, when p21 is produced, there is a concomitant decrease in EGF binding of the transfected 3T3 cells, while the changes in cyclic AMP-mediated hormone response is less pronounced. The ras-transformed cells are known to have an elevated level of diacylglycerol, whether the loss of EGF binding is a consequence of activation of protein kinase C is being studied. This finding is consistent with our previous observation that in a ras-transformed 3T3 cell line, EGF binding is markedly reduced. Since NIH-3T3 cells are relatively undifferentiated, it is desirable to examine the effect of inducible ras gene in a more differentiated cell line. We have obtained plasmids, containing glucocorticoid-inducible normal and viral ras, from others. MDCK cells have now been transfected with these plasmids. We are currently selecting clones expressing the most highly inducible ras gene. Once these transfected clones are identified, we will attempt to examine the temporal relationship between the expression of ras product and the reduction of EGF binding, the elevation of phosphoinositol and the changes in hormone response.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
 Z01 DK 15005-13 LCDB

PERIOD COVERED
 October 1, 1987 to September 30, 1988

TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders.)
 Regulation of Adipocyte Metabolism

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

P.I.:	Constantine Londos	Research Chemist	LCDB:NIDDK
Others:	Soraya Naghshineh	Senior Staff Fellow	LCDB:NIDDK
	John J. Egan	Staff Fellow	LCDB:NIDDK
	Andrew S. Greenberg	Medical Staff Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

I.A. Simpson, S.W. Cushman, MCNEB:NIDDK; C.L. Saxe, A.R. Kimmel, LCDB:NIDDK;
 K.P. Huang, ERRB:NICHHD; A.M. Spiegel, M.D., NIDDK; K.B. Seamon, CDB:DB; R.A.
 Kahn, LBC:NCI

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Membrane Regulation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

4.0

PROFESSIONAL

3.5

OTHER:

0.5

CHECK APPROPRIATE BOXES)

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

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

We have examined various aspects of hormonal control of adipocyte metabolism with isolated rat epididymal adipocytes as a model system. (A) In 32-Pi-loaded cells, the phosphorylation of a 65 KDa protein, found associated with the lipid fraction of adipocyte homogenates, was examined under conditions of unchanging, steady-state cAMP-dependent protein kinase (A-kinase) activity. Steady-state phosphorylation was achieved following a pulse, or overshoot, of phosphate incorporation, indicating that increases in A-kinase are accompanied by increased phosphatase activity, and that the concerted action of both kinase and phosphatase provide the cell with a means to produce graded responses to graded increases in cellular cAMP. In a manner independent of A-kinase activity, insulin also leads to the removal of phosphate from this protein. Insulin stimulates the phosphorylation of an abundant 62 KDa protein, also located exclusively in the lipid fraction; phosphorylation of this protein is abolished by increased A-kinase activity. Such data reveal a tight interplay, or crosstalk, between adenylate cyclase-linked receptors and the insulin receptor. (B) A high affinity antibody against hormone-sensitive lipase (HSL) was raised and used to probe a fat cell cDNA library. Also, a probe for the HSL gene has been produced from a peptide sequence derived from HSL purified in this laboratory. (C) Analysis of fat cell G proteins showed that fat cells contain 3 different subspecies of Gi and 2 different Gs proteins, all located primarily on plasma membranes. However, intracellular membranes contain a large "Gs-like" protein of 55 KDa that is ADP-ribosylated by cholera toxin, both in vivo and in vitro, and recognized by affinity purified antibodies against Gs.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15100-18 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Protein Nucleic Acid Interactions: Chromatin Structure and Function

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

P.I.:	R. T. Simpson	Laboratory Chief	LCDB:NIDDK
Others:	S. Chambers	Senior Staff Fellow	LCDB:NIDDK
	A. Dranginis	Senior Staff Fellow	LCDB:NIDDK
	R. Morse	NRSA Fellow	LCDB:NIDDK
	R. Parker	Senior Staff Fellow	LCDB:NIDDK
	D. Pederson	Senior Staff Fellow	LCDB:NIDDK
	C. Szent-Gyorgyi	Senior Staff Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

R. and L. Angerer, University of Rochester
T. Richmond, ETH, Zurich, Switzerland

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

6.0

6.0

0.0

CHECK APPROPRIATE BOX(ES)

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

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

We use a variety of experimental systems to address fundamental questions relating to chromatin structure, transcriptional regulation of genetic activity, and differentiation and development. Several studies have been carried out using a method which allows isolation of unique yeast genes, as chromatin, in the expressed and repressed states. We have found that the chromatin structure of the HSP26 gene is essentially identical in the genomic, single copy, and the plasmid, amplified, forms. Surprisingly, the structure is unaffected by heat shock induction of transcription. This suggested that the trans-acting regulatory factors involved in heat shock gene expression are constitutively associated with the gene; minichromosome isolation and examination of proteins present support this hypothesis. Other studies of yeast plasmid chromatin involve the 5S rRNA gene and genes regulated by the MATa locus.

We have studied the role of chromatin structure in transcription using the 5S rRNA gene of *Xenopus*. The gene, or variants, was associated with histones and transcribed in egg, oocyte, or germinal vesicle extracts. A novel method for evaluating the role of positioned nucleosomes was developed to further define the role of chromatin structure in transcriptional regulation. Our data suggest that nucleosome formation inhibits binding of polymerase and TFIIIA. Elongation of transcription is partially inhibited by the presence of a nucleosome. Differences in nucleosomes may relate to transcription through the histone complex; yeast and chicken histones constrain DNA to very different extents. This is of interest due to the fact that yeast chromatin is about 10 times more actively transcribed than that of most larger eukaryotic cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15102-28 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Study of Ribonuclease and its Inhibitor from *Bacillus amyloliquefaciens*

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

P.I.: Robert W. Hartley

Research Physicist

LCDB:NIDDK

Others: Peter FitzGerald

Staff Fellow

LCDB:NIDDK

COOPERATING UNITS (if any)

C. Hill, Dept of Chem., UCLA

J. Garnier, Lab. Biologie Physicochimique, U. Paris Sud, France

A. R. Fersht, Dept. of Chem., Imperial College, London

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS.

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Two proteins, barnase, the extracellular ribonuclease of *Bacillus amyloliquefaciens*, and barstar, its intracellular inhibitor, are used as a model system for the study of protein folding and protein-protein interactions. Barnase is one of an homologous group of ribonucleases occurring in both prokaryotes and eukaryotes.

Recombinant DNA techniques are being applied to the project with three major aims: (1) to facilitate production; (2) to examine the structural and control sequences of the genes; and (3) to tailor specifically designed modifications in the sequences to test theories of protein folding.

The lethal effect of the cloned wild-type barnase gene in either *E. coli* or *B. subtilis* can be repressed by expression of the barstar gene placed on the same plasmid. *E. coli* plasmid vectors have been devised for both proteins and both can now be obtained essentially pure in 100 mg quantities.

DNA and amino acid sequences are known for both, and the x-ray structure of barnase has been refined. Preliminary studies suggest that the structure of barstar may be solvable by NMR.

At least one inactive barnase mutant forms a normal complex with barstar.

A simple procedure for oligonucleotide-directed mutation using only plasmid DNA has been devised and applied to both genes.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15200-28 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies on Folic Acid (Dihydrofolate Reductase) and Vitamin A

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

P.I.: Bernard T. Kaufman

Research Chemist

LCDB:N1DDK

Others: John Bieri

Scientist Emeritus

LCDB:N1DDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Nutritional Biochemistry Section

INSTITUTE AND LOCATION

N1DDK:NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

We are continuing our studies on dihydrofolate reductase (DHFR) since the function of this enzyme is not only essential for cellular proliferation, it is also the site of action of Methotrexate, a key drug in the treatment of a variety of human scourges. Theoretically, the reduction of dihydrofolate by DHFR should yield linear reaction kinetics. This is not the case with the bacterial reductases where the initial rate is characterized by a significant lag. This phenomenon is designated as slow transient kinetics and the enzyme is said to exhibit hysteresis if preincubation with substrate eliminates the lag. However, under our analytic conditions DHFR from various animal sources, chicken and beef liver as well as certain tumor cells, exhibit linear reaction rate to within 85% of completion. On the other hand, the characterization of sheep liver DHFR is consistently complicated at all stages of purification by non-linear initial reaction lags. Although this behavior is suggestive of hysteresis, the initial lag cannot be eliminated by preincubation with either substrate. Neither pH or buffer composition affects the lag. However, increasing ionic strength diminishes this effect and at 0.03 M KCl the rate becomes essentially linear. Increasing the salt concentration also markedly increases the catalytic activity. Thus, at 0.8 M KCl, the rate of sheep liver DHFR is increased 6-fold. Similar effects are noted with high concentrations of chaotropic agents such as urea and guanidinium salts.

Studies on vitamin A have shown that individuals vary widely in the efficiency of their carotenoid adsorption. In addition, a high negative correlation between the baseline plasma beta carotene and the maximum percentage increase after a dose of pure beta carotene together with the observation that the proportionate increase in plasma concentration is lower at the higher dosage level suggest that carotenoid absorption and transport have limiting characteristics. Individuals have a relatively constant steady-state pattern of plasma carotenoids.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15302-18 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders.)

Biochemical Studies of Hepatic and Intestinal Function

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

P.I.: Herbert G. Windmueller Research Chemist LCDB:NIDDK
Others: Albert E. Spaeth Chemist LCDB:NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Nutritional Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15400-14 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Hormones, Lipoprotein Lipase and Lipid Metabolism

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

P.I.:	Robert O. Scow	Chief, Endocrinology Section	LCDB:NIDDK
Others:	Hiroshi Masuno	Visiting Fellow	LCDB:NIDDK
	E. Joan Blanchette-Mackie	Research Biologist	LCDB:NIDDK

COOPERATING UNITS (if any)

Dr. Thomas Olivecrona, Dept. of Physiol. Chem., Univ. of Umea, Sweden
 Dr. Kazuhiro Oka & Dr. W. Virgil Brown, Medlantic Research Foundation,
 Washington, DC

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Endocrinology

INSTITUTE AND LOCATION

NIDDK NIH Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL

OTHER:

2.75

1.75

1

CHECK APPROPRIATE BOX(ES)

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

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

Mice born with combined lipase deficiency (cld/cld) develop extreme hypertriglyceridemia and die within 3 days. They have very low levels (less than 5% normal) of lipoprotein lipase and hepatic lipase activities, their tissues are virtually devoid of fat, and 95% are tailless. Studies in vivo showed that brown adipose and other tissues of cld/cld mice synthesize normal sized lipoprotein lipase protein, but the enzyme is inactive and not transferred to capillaries, the normal site of action of this enzyme. The deficiency is caused by a recessive mutation (cld) in the T/t complex of chromosome 17.

Cells cultured from brown adipose tissue of 1-d old cld/cld and unaffected mice readily differentiated into brown adipocytes. Unaffected adipocytes contained and released lipoprotein lipase activity to the medium, whereas cld/cld adipocytes contained very little lipase activity and released none to the medium. cld/cld adipocytes contained 2 x normal amounts of lipoprotein lipase protein but, unlike unaffected cells, they released none to the medium. These findings demonstrate that cld/cld brown adipocytes synthesize an inactive nonsecretable form of lipoprotein lipase.

Lipoprotein lipase was found by immunocytochemistry in both endoplasmic reticulum and Golgi of unaffected brown adipocytes, whereas it was found only in endoplasmic reticulum of cld/cld adipocytes. Since lipoprotein lipase synthesized by cld/cld tissues is normal sized, these findings suggest that lipoprotein lipase synthesized in cld/cld adipocytes is glycosylated in endoplasmic reticulum, receiving a high-mannose type oligosaccharide. However, the lipase is not transported to the Golgi apparatus where normally the oligosaccharide component would be modified and the enzyme would become active and could be secreted by the cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15401-16 LCDB

PERIOD COVERED

October 1, 19887 to September 30, 1988

TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders.)

Transport of Lipids, Hormones & Enzymes in Tissues, Cells & Membranes

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

P.I.:	Robert O. Scow	Chief, Endocrinology Section	LCDB:NIDDK
Others:	E. Joan Blanchette-Mackie	Research Biologist	LCDB:NIDDK
	Carmen Mateo	Visiting Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.25

PROFESSIONAL

1.25

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Fatty acids (FA) and monoacylglycerol (MAG) produced during absorption of dietary fat are generally thought to be solubilized and transported in intestinal contents as micelles formed with bile salts. Recent findings elsewhere indicate that lamellar structures coexist with mixed micelles and could be involved in transport of lipolytic products into intestinal cells. We investigated this possibility by studying, with a flow-through chamber and phase microscope, effects of various substances on hydrolysis of long chain triacylglycerol (TAG) by pancreatic lipase and transport of lipolytic products in aqueous media at pH 7.4. We found that FA and MAG produced by lipolysis were insoluble in aqueous media at pH 7.4. Calcium enhanced lipolysis but had no effect on release of lipolytic product to the media, whereas sodium taurodeoxycholate (TDC) caused release of lipolytic product to the media without affecting lipolysis. Release of lipolytic products by TDC, however, was slow with only 1/3 released in 30 min. TDC enhanced by 60% the stimulatory effect of calcium on lipolysis.

Our morphological observations indicated that FA and MAG formed bilayered (lamellar) structures before they were dispersed by KTDC in the aqueous media. Findings elsewhere in patients with bile salt deficiencies showed bile salts are not necessary for digestion and absorption of dietary fat. Earlier studies showed in rats and fish that dietary fat droplets undergoing lipolysis were attached to the luminal surface of intestinal cells. Thus, we propose that FA and MAG may be transported from dietary fat droplets into intestinal epithelial cells, in the presence of bile salts, by lateral flow in an interfacial continuum composed of the monolayer surrounding the droplets and the outer leaflets of cell membranes, a process similar to that observed for transport of lipolytic products from chylomicrons in plasma to the interior of parenchymal cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15404-04 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Ultrastructural Immunocytochemistry of Lipid Metabolism in Cultured Cells & Tissues

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

P.I.:	E. Joan Blanchette-Mackie	Research Biologist	LCDB:NIDDK
Others:	Robert O. Scow	Chief, Endocrinology Section	LCDB:NIDDK
	Hiroshi Masuno	Visiting Fellow	LCDB:NIDDK
	Nancy K. Dwyer	Biologist	LCDB:NIDDK

COOPERATING UNITS (if any)

Dr. Thomas Olivecrona, Dept. Physiol. Chem., Univ. of Umea, Sweden; Dr. Carl Alving, Dept. Membrane Biol., Walter Reed Army Inst. of Res., Washington, DC
 Dr. Peter Pentchev, Div. Metab. Neurol. Br., NINCDS, NIH; Dr. Howard S. Kruth.

LAB/BRANCH Lab. Exptl. Ather., NHLB, NIH

Laboratory of Cellular and Developmental Biology

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIH NIDDK Bethesda, Maryland 20892

TOTAL MAN-YEARS.

2.5

PROFESSIONAL:

1

OTHER:

1.5

CHECK APPROPRIATE BOXES)

- (a) Human subjects
 (a1) Mincrs
 (a2) Interviews
 (b) Human tissues
 (c) Neither

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

Combined lipase deficiency (cld/cld) in mice is characterized by marked functional deficiencies of both lipoprotein lipase and hepatic lipase. We used immunocytochemistry to locate intracellular lipoprotein lipase in cultured brown adipocytes derived from cld/cld mice and their unaffected littermates. Unaffected brown adipocytes treated with monensin, which inhibits glycoprotein transport from Golgi, accumulated lipoprotein lipase within the Golgi complex. In contrast, cld/cld brown adipocytes contained intracellular lipoprotein lipase distributed in a reticular pattern throughout the cell and this distribution was not altered by monensin treatment. Electron microscopic immunoperoxidase studies showed that lipoprotein lipase was present within the endoplasmic reticulum of cld/cld brown adipocytes. These studies indicate the cld/cld cells synthesize a lipase which accumulates in endoplasmic reticulum and is not transferred to Golgi for further modification. This defect in intracellular processing of lipoprotein lipase results in the inability of cld/cld brown adipocytes to secrete the enzyme.

Type C Niemann Pick (NP-C) disease is an autosomal-recessive neurovisceral lipid storage disorder. Fibroblasts derived from patients with this disease incubated with LDL accumulated intracellularly excessive amounts of unesterified cholesterol. Cytochemical techniques revealed that this abnormal cholesterol accumulation is associated not only with storage of cholesterol in lysosomes but with cholesterol enrichment of the Golgi complex. These findings indicate that components of the Golgi complex play a role in the intracellular translocation of exogenously derived cholesterol and that disruptions of the cholesterol transport pathway at the Golgi may be responsible for the deficiency in cholesterol utilization by NP-C fibroblasts.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15500-28 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Large-Scale Processing of Biological Material

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

P.I.:	Joseph Shiloach	Research Chemist	LCDB:NIDDK
Others:	Jeanne B. Kaufman	Biol. Laboratory Technician	LCDB:NIDDK
	Raphael Fass	Visiting Fellow	LCDB:NIDDK
	Michele van der Walle	NRC Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

0

PROFESSIONAL

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

The Pilot Plant (Biotechnology) Unit combines several different types of activities. It is responsible for the large-scale production of bacteria, mammalian cells and biologically active compounds from various sources. Parallel to this activity, it is conducting process development work associated with these preparations in order to be able to execute them efficiently. In addition, the unit is carrying on research work not necessarily associated with a current project, but work that has long-term implications for the unit's performance.

During the last year, the unit carried out 150 different large-scale preparations, including microorganism growth in volumes from 10 to 300 liters, mammalian cell growth up to 50 liter volumes and processing of various biological materials.

Development work was done in the optimization of E. coli growth using the fed batch technique and oxygen-enriched air yielding 100 g/liter wet weight of biomass. A new method for the purification of Exotoxin A from Pseudomonas aeruginosa was developed.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15503-07 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1987

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

Regulation of Developmental Gene Expression

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

P.I.:	Alan R. Kimmel	Senior Staff Fellow	LCDB:NIDDK
Others:	Charles Saxe	Senior Staff Fellow	LCDB:NIDDK
	Stephen Saxe	Staff Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

3

OTHER:

0

CHECK APPROPRIATE BOXES)

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

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

The growth and differentiation of eukaryotic cells is often modulated by extracellular molecules. Receptors on cell surfaces are stimulated by these signals which can then activate a series of effectors which produce a variety of intracellular second messengers such as cAMP, IP, DAG, calcium and cGMP. Each of these is capable of activating specific protein kinases. Ultimately, the proteins phosphorylated by these kinases are suggested to interact with cellular components to modulate the expression of the eukaryotic genome and promote cell proliferation and cytodifferentiation. We have been studying these processes in Dictyostelium discoideum, an organism whose developmental cycle is controlled by extracellular cAMP. We have established a linkage of the expression of individual gene families with the accumulation of specific intracellular second messengers. Additionally, we have isolated genes encoding components of the signal transduction system. Two genes have been characterized which code for cell-surface cAMP receptor molecules. Data indicate that these receptors traverse the plasma membrane seven times as other receptors which interact with G-proteins. Two genes encode different sized mRNAs which are expressed at different times during the developmental cycle. One is induced during early development; the second is detected during cytodifferentiation. We have also been studying genes for GTP-binding proteins. Two novel genes have been isolated which also exhibit differences in their regulation during development. One is expressed in growing cells and is repressed during development; the other is only expressed in multicellular aggregates.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15506-05 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Control of Gene Expression in Early Mammalian Development

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

P.I.:	Jurrien Dean	Senior Investigator	LCDB:NIDDK
Others:	Margaret Chamberlin	FAES Graduate Student	LCDB:NIDDK
	Li-fang Liang	IRTA Fellow	LCDB:NIDDK
	Sarah Millar	Visiting Fellow	LCDB:NIDDK
	Dwayne Lunsford	Staff Fellow	LCDB:NIDDK
	Anne Baur	Chemist, GS-11	LCDB:NIDDK

COOPERATING UNITS (if any)

Frank Robey, LCDB:NIDR	Satoru Shimizu, Aichi Cancer
Connie Oliver, LMI:NIDR	Research Institute, Nagoya,
Tom Fuerst, Bernard Moss, LVD:NIAID	Japan

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MANYEARS:

5.4

PROFESSIONAL:

4.4

OTHER:

1.0

CHECK APPROPRIATE BOXES)

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

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

The mouse zona pellucida is an extracellular glycocalyx comprised of three sulfated glycoproteins (ZP1, ZP2 and ZP3) which surrounds growing oocytes, ovulated eggs and dividing embryos. One of the proteins, ZP3, has been shown to be the primary sperm receptor and to induce the sperm acrosome reaction. The ZP3 gene is transcribed uniquely in oocytes and transcripts accumulate during the narrow two-week growth phase of oogenesis. We have determined that ZP3 is a single copy gene composed of 8 exons (ranging in size from 92-338 bp) which encompass approximately 8.6 kb of DNA on mouse chromosome 6. We have identified a novel tandem repeat with a unit length of 54 bp which is re-iterated 6 fold, 500 bp upstream of the transcription start site. Studies are currently underway to determine if this repeat or other 5' flanking sequences play a role in the oocyte-specific, developmentally regulated expression of ZP3. The poly-adenylated ZP3 transcript contains short 5' and 3' untranslated regions and a single open reading frame sufficient to code for a core protein of 46,307 daltons. The cleavage of a putative 22 amino acid signal peptide would result in the secretion of a mature core protein of 43,943 daltons. One strategy to investigate the biological functions of ZP3 is to examine the effect of anti-ZP3 antibodies on oogenesis and early development. We have described an anti-ZP3 monoclonal antibody that is effective in inhibiting *in vitro* and *in vivo* fertilization, but has no other effect on oogenesis or early development. This contraceptive effect is long-term (>15 estrus cycles), but eventually reversible. The epitope recognized by this contraceptive antibody has been identified by recombinant DNA techniques as a seven amino acid peptide. Mice vaccinated with this peptide produce antibodies which bind to endogenous intra-ovarian oocytes and the biological effect of these antibodies in mice is now being investigated. Because the ZP3 gene is conserved from mouse to man, this contraceptive strategy may be widely applicable among mammals.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15508-01 LCDB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Chromatin Structure in Regulation of Mammalian Developmental Gene Expression

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

P.I.: Ann Dean Research Chemist LCDB:NIDDK
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COOPERATING UNITS (if any)

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Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Understanding how gene expression is controlled in a temporal and tissue specific manner is a basic problem in developmental biology. The individual members of the human globin family are temporally regulated so as to bring about the sequential production of embryonic, fetal, and adult hemoglobins during ontogeny. We are interested in the changes in chromatin structure that these genes undergo when they are activated during development. We have used the electrophoretic mobility shift assay to detect interactions between putative trans-acting regulatory factors present in the nuclei of cells actively transcribing globin genes and cis-acting sequences flanking the epsilon and gamma globin genes. We detected the formation of several complexes between K562 nuclear proteins and a fragment of the human epsilon globin promoter. DNase I footprinting and exonuclease protection assays suggested some of these interactions occurred at elements common to many regulated eukaryotic genes, i.e., at the CCAAT and ATA sequences and over the major transcription initiation site. One strong binding site for the general eukaryotic transcription factor Sp1 was observed in the epsilon-globin promoter. The proteins participating in these interactions could be partially resolved on DNA-agarose columns, however, none was erythroid specific. Strong binding sites for an erythroid specific protein present in K562 nuclear extracts were located in the 3' flanking regions of the epsilon-, gamma-, and beta-globin genes. The gamma- and beta-globin sites corresponded to regions which have been shown to possess enhancer activity for their respective genes. These sites are situated in regions of DNA which display tissue and developmental stage specific DNase I hypersensitive sites when the genes are expressed. However, the factor is present in erythroid cells of different developmental stages. We will pursue studies aimed at understanding the structural and functional significance of the binding of this factor to DNA.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMISTRY AND METABOLISM
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory conducts research in such apparently disparate areas as differentiation, morphogenesis, endocytosis, endocrinology, membrane transport, detoxication and the physical and chemical behavior of proteins and nucleic acids, and does so by an array of different methods applied to the identified problems. Resolution is being attempted by approaches that stem from enzymology, carbohydrate chemistry, cell biology, biophysics and molecular biology. Although seemingly diverse, there is a common element to each of the subjects summarized here that is appropriate to the Laboratories' designation: biochemical, metabolic and physical approaches are being brought to bear on major problems encompassed by the Institute's charge. It is the close proximity of experienced investigators from diverse scientific disciplines, discussing their individual fields with each other, that provide synergistic effects for the resolution of the questions under investigation.

I. PHYSICAL ASPECTS OF MEMBRANES, TRANSPORT AND PROTEINS

The more physical attributes of proteins and lipids, as well as their interaction in membranes and function in transport, is the concern of several groups in the Laboratory.

Ionic channels and other proteins with aqueous cavities

Proteins, especially those designed to transport ions across channels, are sensitive to osmotic stress from materials unable to enter their aqueous cavities. The ability to exert osmotic stress to measure the amount of water that goes in or comes out when a protein, particularly a trans-membrane ionic channel, opens or closes is now being used. Some 20 to 40 water molecules were found to enter a typical potassium channel of the squid giant axon when it opens under electrical stimulation. These measurements, on whole axon preparations, suggest that significant changes in protein structure must occur during channel opening. Such large changes are quite different from what is usually imagined to be channel "gating".

A new method has been developed for selecting and separating vesicles containing single channels and for incorporation into artificial systems. This development is enabling more controlled protein reconstitution into artificial bilayer membranes for single-channel study.

Major effort has been successfully expended in designing computer hardware and software to allow high-speed data acquisition while recording channel electrical activity, concurrently analyzing results and putting the variation of experimental conditions under computer control. This system, a prototype for personal computer laboratory use, also makes possible better connection with powerful mainframe equipment for more sophisticated data analyses.

Forces between macromolecules

The actual measurement of forces between large molecules may be expected to teach how these molecules interact to form the functioning units of a living cell. The results of such direct measurements are quite different from earlier expectation. For example, it has been learned that in the important last few nanometers in which molecules approach contact, interaction is dominated by a continuously varying work of removal of water solvent from their surfaces. Between phospholipid bilayer membranes, these important "hydration forces" are exquisitely sensitive to very small changes in the composition of the lipid polar groups, changes that are under the biochemical control after formation of the membrane structure.

Success has recently been attained in measuring not only the force vs. separation of molecules but also the change in their thermal motion during mutual approach. Outside the 1 nanometer range of direct hydration repulsion, it appears that macromolecules move or undulate to repel by the "steric" action of molecules colliding. However, these collisions never involve molecular contact but rather occur through long-range forces between molecular surfaces. The result is a form of interaction qualitatively different from any that has been assumed to be responsible for molecular assembly.

One can now use these data to see how the measured forces act at the functional level of controlling the contact and fusion of membranes as in a secretory process, or determining the packing of DNA or other long molecules, or perturbing the rearrangements of protein structure that effect the "gating" of trans-membrane ionic channels, or even effect the activity of enzymes whose function depends on particular forms of packing components. Each of these processes are being investigated.

Histamine release from beige mouse mast cells

As a consequence of fusion of an intracellular granule with the plasma membrane, an exocytotic pore (which characterizes the omega-figure seen in electron micrographs of secreting cells) connects the granule interior with the extracellular medium. With the use of the patch clamp technique and mast cells from beige mice, small fusion pores have been discovered which initiate exocytosis. These pores are much smaller than the limit of electron microscopy. These techniques allow following the rapid enlargement of this pore for every fused granule. Large, stepwise increases in capacitance are recorded as the result of fusion of large, individual secretory granules to the plasma membrane. Pore sizes are derived from the impedance analysis of the equivalent circuit.

An analysis of hundreds of such fusion events show rich and varied kinetics, suggestive of a dynamic fusion pore structure. The widening can be rapid or slow, monotonic or fluctuating. Thus the exocytotic pore has fundamental differences from stable membrane channels and gap junctions, and quick, fluid lipid fusion. A protein/lipid complex is envisioned as playing a role.

Cell-cell fusion

Initial events in infection by enveloped viruses, such as influenza, rabies, herpes and HIV, involve binding of virus to host cell plasma membrane followed by fusion to the plasma membrane or internalized membrane after receptor-mediated endocytosis. A recently developed series of real-time fluorescence probe methods have allowed following the fusion of viruses to cultured cells or human red blood cells (RBC) to cells expressing viral spike glycoproteins (SGCs) on their extraplasmic surface. The results of kinetic analysis of these interactions suggest that the membrane rearrangements of proteins and lipids necessary for fusion first allow lipid exchange followed rapidly by establishment of one or more fusion pores, which allow exchange of soluble molecules. By directly imaging the fluorescent dyes with image enhanced video light microscopy, one can analyze the spatial redistribution of the fluorescent probe between the fusing cells. Methods were developed for using low light image enhanced video microscopy of live cells to analyze a series of objectives concerned with early events in viral protein-mediated membrane fusion. Fusion appears to be established by the viral fusion protein forming a pore which gates the passage of molecules between the RBC and target cell according to size and charge. It is hoped to establish a molecular "time table" which correlates the movement of membrane and cytoplasmic molecules during the fusion process. Using a combination of patch clamp and videomicroscopy, one can also correlate membrane and core probe movements with pore formation.

Thermodynamic and kinetic studies of protein structure

This laboratory is engaged in studies on protein structure and the mechanism by which a protein molecule, which is synthesized as a random coil, can fold into a specific secondary and tertiary structure, without any external help. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight 39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of it's first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Both proteins are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. After such unfolding, pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. The interest is in the mechanism of this refolding reaction and on the influence of the change in sequence on the behaviour of the two proteins. Using techniques such as ultra-violet, circular dichroic and fluorescence spectroscopy, together with chemical modification and peptide chemistry, the structures of the native and unfolded species have been characterized. Using rapid kinetic techniques, such as stopped-flow and T-jump, intermediate, partly folded forms have been detected in the folding reaction, their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.

II. ENZYMES IN MORPHOGENESIS AND DISEASE

Several groups are active in this broadly designated area which includes enzymes operating during the formation of organelles in yeast, the lesion in the gene for an enzyme whose absence leads to Tay-Sachs disease, and the enzymes that appear to be responsible for the hepatocarcinogenicity of a group of arylamines.

Morphogenesis

Transformation of Schizosaccharomyces pombe with the previously cloned putative chitin synthetase 2 gene resulted in expression of the synthetase in S. pombe, thus showing that the cloned gene is the structural gene for the enzyme. Disruption of the gene in Saccharomyces cerevisiae led to inability of the yeast cells to form a septum and divide. This result provided the first unequivocal evidence that chitin synthetase 2 is essential for the formation of septum chitin and that blocking of synthesis of a cell wall component results in death of a fungal cell.

Chitin synthetase 1 is not required for septum formation. Under certain conditions, however, a chitinase that normally acts in cell separation may become exceptionally active, with a potential for damaging the cell wall and causing lysis. Recent results suggest that under those circumstances, the presence of chitin synthetase 1 leads to increased synthesis of chitin and prevents cell lysis, i.e. chitin synthetase 1 appears to function as an emergency or repair enzyme.

The enzyme that catalyzes the biosynthesis of the major structural polysaccharide of the yeast cell wall, beta(1-3) glucan synthetase, was previously separated into two fractions, a soluble GTP-binding regulatory component and a membrane-bound, presumably catalytic, component. By using a combination of detergents, the catalytic fraction has been solubilized. Each component is now available in soluble form and free from the other one.

Genetic lesions in Tay-Sachs disease

Ashkenazi Jewish patients with classic Tay-Sachs disease were found to lack mRNA coding for the alpha-chain of beta-hexosaminidase, but retain a grossly intact alpha-chain gene indicating the presence of a subtle defect. During the past year, the sequence analysis of the promoter region, exon and splice junction regions and polyadenylation signal area of the mutant gene have been completed. Only one difference was observed between these sequences: at the 5' boundary of intron 12 a guanosine in the conserved splice junction dinucleotide sequence GT had been altered to a cytidine. The alteration is presumed to be functionally significant and to result in aberrant mRNA splicing.

An assay was developed which utilized the polymerase chain reaction to amplify the region encompassing the lesion to screen patients and carriers for the splice junction mutation. In two Ashkenazi patients tested only one allele harbored the splice junction mutation. Others were negative for this mutation, and 30% of the obligate heterozygote carriers tested displayed the mutation. These results demonstrate that at least two lesions underlie the classic form of Tay-Sachs disease in the Ashkenazi Jewish population. At present, alpha-chain genomic clones from an Ashkenazi Jewish patient negative for the splice junction lesion are being isolated and are being sequenced in the critical region of this gene to determine the second mutation.

Carcinogenicity of certain arylamines

Two isoenzymes of amine N-methyltransferase from rabbit liver are available in homogeneous form and have been characterized with regard to specificity. The enzymes have overlapping specificity for the transfer of a methyl group from S-

adenosyl-L-methionine to any one of a very large number of amine acceptors. Acceptor molecules include those of very different carbon skeleton among which are aliphatic, aromatic and heterocyclic compounds that are primary, secondary and tertiary amines.

It has now become possible to evaluate the role of methylation in the formation of certain hepatocarcinogenic amines. Hepatocarcinogenicity for compounds such as benzidine, N-methylazobenzene and some other aminobiphenyls is well established. Based on work with purified enzymes, the N-methyltransferase and a microsomal flavin-containing monooxygenase, it was concluded that carcinogenicity of such aromatic amines required methylation followed by N-oxidation of the resultant N-methyl derivative; the cytochrome P-450 system did not appear to be involved. Thus benzidine and 4-aminobiphenyl are both methylated and carcinogenic. 4-Aminoazobenzene is neither methylated nor carcinogenic, although the methylated species, 4-methylaminoazobenzene is carcinogenic. All three carcinogenic monomethyl arylamines yield N-oxidized products with the flavin oxygenase. 2-Aminobiphenyl is neither a substrate for the transferase nor does it act as a tumor inducer. It would appear that N-methylation, followed by oxidation with the flavoprotein system, is the means for producing carcinogenic derivatives of the aminobiphenyls.

III. INTERACTION OF DNA AND NUCLEAR PROTEINS

The interaction of specific proteins with DNA is probably the major regulatory pattern of growth and differentiation. Two groups are addressing these interactions, one by a strictly physical technique and the other by molecular genetics.

Mapping the topology of DNA

Major emphasis has been placed on identifying and overcoming the problems associated with photochemical electric dichroism. This technique is a hybrid of classical electric dichroism and the methodologies developed by molecular biologists for examining DNA-protein complexes. The end result will be a technique that can sensitively and selectively map the topology of DNA folding or wrapping in specific DNA-protein complexes of biological importance. The problems we have encountered primarily result from the very large electric fields applied across the sample and the very intense UV laser light that must be employed. Electrode poisoning is the primary effect. A greater emphasis on electrode cleaning and preparation appear essential for optimal results. Additionally, buffer conditions for the dichroism experiment are far more critical for this type of experiment than classical dichroism because of nonspecific background nicking of DNA. Improvements in signal quality and differences observed in photodamage probability between oriented and unoriented reconstituted nucleosomes indicate that the technique will work and become a dominant structural method for protein-DNA complexes with sensitivities and specificities comparable to footprinting.

A new computer-linked data collection system has been installed in an electric birefringence and dichroism apparatuses. The LeCroy system gives a greater time resolution and makes data analysis faster and more convenient. Work on other projects can now proceed at a faster rate. Work on bent DNA fragments has been expanded to include oligo A tract sequences that show interesting flanking sequence properties. Initial results on bipolar myosin filaments indicate a great deal of internal motion and that this motion is different for

phosphorylated and unphosphorylated myosin. Building on previous results from this laboratory, construction of dynamical models for the bipolar filament is about to begin.

Tissue specific and hormone regulated gene expression

The molecular basis of mammary specific and hormone regulated gene expression is being studied through analysis of cis-acting regulatory elements of the mouse whey acidic protein (WAP) gene using in vitro systems and transgenic animals. It was shown that nuclear proteins from mammary epithelial cells form a multiple nucleoprotein complex with the WAP gene promoter and upstream region. The WAP and alpha-lactalbumin gene promoters share sequence motifs which are recognized by nuclear proteins, suggesting that they play a role in the regulation of these genes. Using transgenic animals the WAP gene promoter was shown to target the expression of a foreign gene to the lactating mammary gland suggesting that it contains regulatory elements governing WAP gene expression in the intact animal. Using this mammary expression system it is also possible to produce human proteins in the milk of transgenic animals which provides an alternative way for isolating rare proteins of scientific and pharmaceutical value.

The structure and function of the control region of the human cytomegalovirus (HCMV) immediate early 1 (IE1) gene has been studied using in vitro systems which partially mimic the regulation observed in vivo. The IE1 enhancer dependent transcriptional stimulation in vitro is about 25-fold and involves the binding of transcription factors. The sequence structure of the enhancer region, which extends from nucleotide -65 to -530, exhibits a highly modulate complexity and is recognized by several nuclear proteins. Using in vitro transcription competition assays with individual target sequences for enhancer binding factors, it has been shown that transcription factors bind to at least five specific enhancer sequences and mediate the activity of the IE1 gene enhancer in vitro.

Cell specific activity of elements within the HIV-LTR

Information has been obtained about promoter, enhancer and repressor elements that regulate human cytomegalovirus (HCMV) genes and on the relationship between gene expression and binding of transcription factors to cis-acting elements. In extension of this work, transcriptional regulation from the HIV-1 long terminal repeat (LTR), which contains control elements required for viral activation, has been examined. In vitro transcription systems were established from lymphoid and nonlymphoid cell lines and accurate initiation of transcription from the HIV LTR was observed. New findings show that the in vitro systems can direct enhancer-dependent transcription from the HIV-1 LTR in several cell types, including T-cells, B-cells and epithelial cells. Although regulatory elements within the enhancer core sequences are preferentially active in lymphoid cells, only a limited host cell restriction of HIV transcription initiation in vitro was observed. The possibility of transcription factors binding to the enhancer sequences and mediating transcriptional stimulation was investigated. Using sensitive binding and competition assays it was shown that proteins bind to the HIV-1 enhancer core sequences and that protein-DNA interaction is necessary for enhancer dependent transcriptional stimulation.

IV. PROTEIN SORTING AND GLYCOPROTEINS

Central to modern biology is the issue of mechanism for the movement of macromolecules, glycoproteins in particular, not only into and out of the cell but also into specific organelles. These plural mechanisms are being sought from the disciplines of somatic cell genetics, molecular biology carbohydrate chemistry, endocrinology and biochemistry. The implications of the work extend from cell biology to applications in thyroid pathobiology and the AIDS virus.

Endocytosis, secretion and compartmentalization in mutant CHO cells

The intent is to dissect the processes of endocytosis, glycoprotein biosynthesis and sorting through isolation and analysis of mutants. Previously most CHO cell endocytosis mutants have been shown to fall into two genetic complementation groups, End1 and End2; both classes of mutants are defective in endosomal, but not lysosomal, acidification. Having identified a candidate for the End2 protein, a novel preparative, three-dimensional gel procedure was developed for purification of membrane proteins in quantities sufficient for immunization.

To obtain new classes of mutants an isolation procedure for cells defective in lysosomal acidification was devised. Exploiting the quenching of fluorescein at acidic pH, cells were screened for exhibition of above normal fluorescence after pulse-chase labeling with fluoresceinated dextran (Mr 70,000). One such mutant accumulates dextran in large non-acidic vacuoles; based on functional assays, its endosomal acidification is unimpaired.

Analysis has continued of a mutant Ltk-cell (LEFIC) which is cross-resistant to toxins but has normal endosomal function. The principal defect in LEFIC appears to involve movement of membrane proteins from late Golgi regions to the plasma membrane. Oddly, delivery of membrane proteins in LEFIC is more severely affected than is secretion of soluble proteins.

To further characterize mutants defective in early steps in the pathway of N-linked glycosylation, an in vitro system was developed for biosynthesis, translocation and elongation of lipid-linked oligosaccharides in intact microsomal vesicles. Conditions for measurement of translocation of lipid-linked Man5GlcNAc2 (the intermediate believed to move from external to luminal faces of the ER) without elongation of the oligosaccharide have been established.

Modulation of the asialoglycoprotein receptor

Studies on the mechanism of ligand-induced modulation of the asialoglycoprotein receptor on hepatoblastoma cells, HepG2, have shown the resultant down-regulation to arise from an alteration in the biosynthesis of sialic acid. Examination of the individual enzymatic reactions leading to the formation of sialic acid in vitro revealed no significant difference in the biosynthetic capacity of the control versus the modulated cells. This finding contrasts with the drastic reduction in synthesis exhibited by intact cells and is suggestive of a broader mechanism for the regulation of cell-surface sialic acid.

The nuclear envelope in intracellular protein sorting

The proper sorting of proteins to the cell nucleus has been shown to play a key role in the regulation of cell growth and development. When chemically coupled to the large fluorescent protein B-phycoerythrin, synthetic peptides derived from the amino acid sequence of the SV40 Large T antigen specifically target the protein conjugate to the nucleus. Transport of such conjugates across the nuclear envelope was demonstrated after microinjection into cultured cells or in an *in vitro* import assay using rat liver nuclei. Transport was time, temperature and energy dependent; only conjugates containing the localization sequence were properly transported. Monoclonal antibodies have been generated which bind specifically to this sequence. These antibodies bind to intact T antigen, and to other nuclear proteins, suggesting that the sequence is normally exposed on the outer surface of molecules to be transported into the nucleus. A nuclear localization signal has also been identified in the primary structure of the human glucocorticoid receptor. A synthetic peptide of 10 residues derived from the sequence of this receptor are sufficient to direct transport of large molecules into the nucleus. Attempts are being made to interrupt nuclear targeting of the glucocorticoid receptor.

The nuclear pore complex traverses the nuclear envelope and mediates uptake into the nucleus. Proteins bearing cytoplasmically oriented, O-linked GlcNAc are components of the nuclear pore complex. The nuclear pore glycoproteins can be selectively labelled using the lectin wheat germ agglutinin. This lectin reversibly blocks both import into the nucleus and the export of RNA from the nucleus to the cytoplasm. These findings raise the exciting possibility that cytoplasmic glycosylation may be involved in the assembly or functioning of the nuclear pore. Monoclonal antibodies have been raised against these nuclear pore glycoproteins thus allowing their purification. Sequence information from the major nuclear pore protein was obtained and this protein has been molecularly cloned. The structure and function of the nuclear pore protein is currently being probed using recombinant DNA technology.

Intracellular traffic in HIV infection

An investigation of CD4, the presumed T-cell receptor for the human immunodeficiency virus, has shown that surface expression of this protein is blocked by tunicamycin, a potent inhibitor of glycosylation, under conditions where alternate surface receptors are unaffected. Initial studies employing acute lymphoblastic leukemic cells have been extended by the successful transfection of a plasmid containing the cDNA for CD4 into Chinese hamster ovary cells. Subsequent cotransfection with another plasmid containing the multiple drug resistant gene, has permitted the isolation of stable clones expressing large amounts of CD4. Preliminary data on this material indicates the presence on CD4 of complex or multi-antennary hybrid oligosaccharides.

A targeting sequence has been identified which is sufficient to allow protein molecules as large as 0.5×10^6 to pass through the nuclear envelope. This has been assessed in our laboratory by chemically coupling peptides having the nuclear localization sequence to a highly fluorescent cytoplasmic protein. The sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Cys is sufficient to target these conjugates to the nucleus. The *tat* and *art* gene products of HIV have sequences very similar to this targeting domain. The approach taken is to chemically synthesize peptides corresponding to the *tat* sequence and to examine the ability of this peptide to confer nuclear localization. In addition to these

studies, the structure of the nuclear pore complex, across which the many nucleocytoplasmic exchange processes must occur, is being examined. The structure of the pore complex is explored using recombinant DNA techniques. The major nuclear pore glycoprotein np62 has been cloned and a large segment sequenced.

Cell regulation by pharmacodynamic and autoimmune agents acting on cell membranes

Structure-function relationships in the mechanisms by which glycoprotein hormones (thyrotropin), autoantibodies, alpha 1-adrenergic agents, insulin, insulin-like growth factors, bacterial toxins (cholera and pertussis, for example), the anti-viral protective agent, interferon, and interleukins - alone and in combination - interact with and transmit their message through the cell membrane to affect cell function and pathology are being defined. Studies using monoclonal antibodies and the idiotype antiidiotype theory explore the structure of the receptors for these ligands and the importance of these relationships to the expression of thyroid hyperfunction in Graves' disease; to organ-specific autoimmunity (Graves' disease, Hashimoto's disease, and diabetes); to fluid losses in intestinal diarrhetic states; to thyroid storm and the sympathetic overactivity syndrome of tetanus; to the ability of hormones to modulate the oncogenic state; and to the mechanism by which toxins subvert normal mechanisms to impose their pathological effects. Studies continue to evaluate the role of different signal transduction mechanisms - cAMP, Ca/phosphoinositide and arachidonate - by these agents for growth and differentiation, e.g. thyroglobulin biosynthesis, thyroglobulin biodegradation to T3 and T4, and the transport of T3, T4, monoiodotyrosine, diiodotyrosine, and other amino acids from the lysosome. The role of phosphate and carbohydrate moieties in thyroglobulin structure and post-translational processing is being investigated. Studies explore lipid regulation of receptor expression, with special emphasis on neuronal and thyroid cell growth and development as well as the hormonal regulation of lipid metabolism, LDL receptor expression, and cholesterol biosynthesis. Work is in progress to clone the TSH receptor and to define its structure and regulatory control at the gene level as is work to define the mechanisms by which TSH, insulin, IGF-I and other ligands regulate gene expression.

Electrochemical ion gradients as a mechanism of cellular message transmission

A primary role of the thyroid cell is to synthesize and secrete the thyroid hormones, thyroxine and triiodothyronine. Taking into consideration the variable intake of iodide, this conversion process is accomplished in a regulated manner and involves numerous steps: concentrated uptake of iodide from the bloodstream across the basal membrane of the cell; vectorial transport of iodide through the cell, efflux of iodide across the apical membrane into the lumen of the thyroid follicle; synthesis of thyroglobulin, vectorial transport of thyroglobulin to the follicular lumen; iodination of thyroglobulin; regulated storage of iodinated thyroglobulin; resorption into the cell; and degradation with reutilization of the iodine and secretion of thyroid hormones into the bloodstream. This process is further regulated by hormones including thyrotropin (TSH), adrenergic agents, insulin, cortisol, insulin-like growth factors, and iodine itself. These hormonal influences regulate the many steps described above and these processes have been the focus of some of the work over several years. Work continues to focus on transport proteins involved in the uptake as well as the efflux of iodide in the thyroid.

The work characterizes the properties of this transport and the hormones and agents that influence, regulate or block these processes. A third direction being pursued is the iodinated intermediates between iodide and thyroid hormones mentioned above. Work includes the chemical modification of thyroglobulin as a means of understanding the role of hormone-rich iodopeptides in the synthesis and release of thyroid hormones from thyroglobulin. The work is being focussed at present on the thyroglobulin from patients with endemic goiter, a condition in which, in the absence of nutritional iodide, the thyroid compensates both in the size and function of the gland to provide the essential thyroid hormones. The work continues to investigate the role of ion fluxes as important early events in the action of hormones, as well as the complex pathway of iodine metabolism in the synthesis and release of thyroid hormones in normal function and pathology of the thyroid.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17001-22 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

The Role of the Carbohydrate Moiety of Glycoproteins in Cellular Activity

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

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TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

3

OTHER:

.5

CHECK APPROPRIATE BOXES)

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

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

Studies on the mechanism of ligand-induced modulation of the asialoglycoprotein receptor on hepatoblastoma cells, HepG2, have shown the resultant down-regulation to arise from an alteration in the biosynthesis of sialic acid. Examination of the individual enzymatic reactions leading to the formation of sialic acid in vitro revealed no significant difference in the biosynthetic capacity of the control versus the modulated cells. This finding contrasts with the drastic reduction in synthesis exhibited by intact cells and is suggestive of a broader mechanism for the regulation of cell-surface sialic acid.

An investigation of CD4, the presumed T-cell receptor for the human immunodeficiency virus, has shown that surface expression of this protein is blocked by tunicamycin, a potent inhibitor of glycosylation, under conditions where alternate surface receptors are unaffected. Initial studies employing acute lymphoblastic leukemic cells have been extended by the successful transfection of a plasmid containing the cDNA for CD4 into Chinese hamster ovary cells. Subsequent cotransfection with another plasmid containing the multiple drug resistant gene, has permitted the isolation of stable clones expressing large amounts of CD4. Preliminary data on this material indicates the presence on CD4 of complex or multi-antennary hybrid oligosaccharides.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17002-18 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Enzymatic Basis of Detoxication

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

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TOTAL MAN-YEARS:

4

PROFESSIONAL:

3.5

OTHER:

0.5

CHECK APPROPRIATE BOXES)

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

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

Two isoenzymes of amine N-methyltransferase from rabbit liver are available in homogeneous form and have been characterized. The enzymes have overlapping specificity for the transfer of a methyl group from S-adenosyl-L-methionine to any one of a very large number of amine acceptors. Acceptor molecules include those of very different carbon skeleton among which are aliphatic, aromatic and heterocyclic compounds that are primary, secondary and tertiary amines. Methylation of the tertiary amines, pyridine for example, results in the formation of a pyridinium ion.

It has now become possible to evaluate the role of methylation in the formation of certain hepatocarcinogenic amines. Hepatocarcinogenicity for compounds such as benzidine, N-methylazobenzene and some other aminobiphenyls is well established. Based on work with purified enzymes, N-methyltransferase and a microsomal flavin-containing monooxygenase, it was concluded that carcinogenicity of such aromatic amines required methylation followed by N-oxidation of the resultant N-methyl derivative; the cytochrome P-450 system did not appear to be involved. Thus benzidine and 4-aminobiphenyl are both methylated and carcinogenic. 4-Aminoazobenzene is neither methylated nor carcinogenic, although the methylated species, 4-methylaminoazobenzene is carcinogenic. All three carcinogenic monomethyl arylamines yield N-oxidized products with the flavin oxygenase. 2-Aminobiphenyl is neither a substrate for the transferase nor does it act as a tumor inducer. It would appear that N-methylation, followed by oxidation with the flavoprotein system, is a means for producing carcinogenic derivatives of the aminobiphenyls.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17003-21 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Polysaccharides in Morphogenesis

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

PI:	E. Cabib	Senior Research Chemist	LBM, NIDDK
Others:	S. DasGupta	Visiting Fellow	LBM, NIDDK
	H.-M. Park	Visiting Fellow	LBM, NIDDK
	A. Sburlati	Visiting Fellow	LBM, NIDDK
	S. Silverman	Senior Staff Fellow	LBM, NIDDK
	J.T. Mullins	Guest Researcher	LBM, NIDDK
	E. Mansfield	Summer Student	LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

5

PROFESSIONAL

5

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

Transformation of Schizosaccharomyces pombe with the previously cloned putative chitin synthetase 2 gene resulted in expression of the synthetase in S. pombe, thus showing that the cloned gene is indeed the structural gene for the enzyme. Disruption of the gene in Saccharomyces cerevisiae led to inability of the yeast cells to form a septum and divide. This result provided for the first time unequivocal evidence that chitin synthetase 2 is essential for the formation of septum chitin and that blocking of synthesis of a cell wall component results in death of a fungal cell.

Chitin synthetase 1 is not required for septum formation. Under certain conditions, however, a chitinase that normally acts in cell separation may become exceptionally active, with a potential for damaging the cell wall and causing lysis. Recent results suggest that under those circumstances presence of chitin synthetase 1 leads to increased synthesis of chitin and prevents cell lysis, i.e. chitin synthetase 1 appears to function as an emergency or repair enzyme.

The enzyme that catalyzes the biosynthesis of the major structural polysaccharide of the yeast cell wall, beta(1-3) glucan synthetase, was previously separated into two fractions, a soluble GTP-binding regulatory component and a membrane-bound, presumably catalytic, component. By using a combination of detergents, the catalytic fraction has been solubilized. Each component is now available in soluble form and free from the other one.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17004-20 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

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

PI: Peter McPhie Research Chemist LBM, NIDDK

COOPERATING UNITS (if any)

Smith, Kline and Beckman (Irwin Chaiken); LMB, NCI (Jane Cheng); Dept. of Biochemistry, Georgetown University (Preston Hensley, Assoc. Prof.); LPD, NIAID (Russell Howard); DCRT (Richard Shrager)

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SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This laboratory is engaged in studies on protein structure and the mechanism by which a protein molecule, which is synthesized as a random coil, can fold into a specific secondary and tertiary structure, without any external help. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight=39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of it's first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Both proteins are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. After such unfolding, pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. I am interested in the mechanism of this refolding reaction and on the influence of the change in sequence on the behaviour of the two proteins. Using techniques such as ultra-violet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, the structures of the native and unfolded species have been characterized. Using rapid kinetic techniques, such as stopped-flow and T-jump, intermediate, partly folded forms have been detected in the folding reaction, their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17008-05 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

The Role of the Nuclear Envelope in Intracellular Protein Sorting

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

PI:	J.A. Hanover	Senior Staff Fellow	LBM, NIDDK
Others:	M. D'Onofrio	Visiting Fellow	LBM, NIDDK
	T. Olson	Special Volunteer	LBM, NIDDK
	M.K. Park	Visiting Fellow	LBM, NIDDK
	C. Starr	I.R.T.A.	LBM, NIDDK
	B. Wolff	Visiting Associate	LBM, NIDDK

COOPERATING UNITS (if any)

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Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.2

PROFESSIONAL:

4.2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

(a1) Minors

(a2) Interviews

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

The proper sorting of proteins to the cell nucleus has been shown to play a key role in the regulation of cell growth and development. When chemically coupled to the large fluorescent protein B-phycoerythrin, synthetic peptides derived from the amino acid sequence of the SV40 Large T antigen specifically target the protein conjugate to the nucleus. Transport of such conjugates across the nuclear envelope was demonstrated after microinjection into cultured cells or in an *in vitro* import assay using rat liver nuclei. Transport was time, temperature and energy dependent; only conjugates containing the localization sequence were properly transported. Monoclonal antibodies have been generated which bind specifically to this sequence. These antibodies bind to intact T antigen, and other nuclear proteins, suggesting that the sequence is normally exposed on the outer surface of molecules to be transported into the nucleus. A nuclear localization signal has also been identified in the primary structure of the human glucocorticoid receptor. A synthetic peptide of 10 residues derived from the sequence of this receptor are sufficient to direct transport of large molecules into the nucleus. Attempts are being made to interrupt nuclear targeting of the glucocorticoid receptor.

The nuclear pore complex traverses the nuclear envelope and mediates uptake into the nucleus. The laboratory has demonstrated that proteins bearing cytoplasmically oriented, O-linked GlcNAc are components of the nuclear pore complex. The nuclear pore glycoproteins can be selectively labelled using the lectin wheat germ agglutinin. This lectin reversibly blocks both import into the nucleus and the export of RNA from the nucleus to the cytoplasm. These findings raise the exciting possibility that cytoplasmic glycosylation may be involved in the assembly or functioning of the nuclear pore. Monoclonal antibodies have been raised against these nuclear pore glycoproteins thus allowing their purification. Sequence information from the major nuclear pore protein was obtained and this protein has been molecularly cloned. The structure and function of the nuclear pore protein is currently being probed using recombinant DNA technology.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17009-03 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Tissue Specific and Hormone Regulated Gene Expression

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

PI: L. Hennighausen Visiting Associate LBM, NIDDK

Others: P.A. Furth Special Volunteer LBM, NIDDK
 P. Ghazal Visiting Fellow LBM, NIDDK
 H. Lubon Visiting Associate LBM, NIDDK
 H.H. Niller Special Volunteer LBM, NIDDK
 C. Pittius Special Volunteer LBM, NIDDK

COOPERATING UNITS (if any)

Integrated Genetics, Framingham, MA (K. Gordon); Laboratory of Molecular Genetics, NICHD (H. Westphal)

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SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

3.5

PROFESSIONAL

3.5

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

The molecular basis of mammary specific and hormone regulated gene expression is being studied through analysis of cis-acting regulatory elements of the mouse whey acidic protein (WAP) gene using in vitro systems and transgenic animals. It was shown that nuclear proteins from mammary epithelial cells form a multiple nucleoprotein complex with the WAP gene promoter and upstream region (1). The WAP and alpha-lactalbumin gene promoters share sequence motifs which are recognized by nuclear proteins, suggesting that they play some role in the regulation of these genes. Using transgenic animals we could show that the WAP gene promoter can target the expression of a foreign gene to the lactating mammary gland suggesting that it contains regulatory elements governing WAP gene expression in the intact animal (2,3). Using this mammary expression system it is also possible to produce human proteins in the milk of transgenic animals which provides an alternative way to isolate rare proteins of scientific and pharmaceutical value (2,3).

The structure and function of the control region of the human cytomegalovirus (HCMV) immediate early 1 (IE1) gene has been studied using in vitro systems which partially mimic the regulation observed in vivo. The IE1 enhancer dependent transcriptional stimulation in vitro is about 25-fold and involves the binding of transcription factors. The sequence structure of the enhancer region, which extends from nucleotide -65 to -530, exhibits a highly modulate complexity and is recognized by several nuclear proteins (4). Using in vitro transcription competition assays with individual target sequences for enhancer binding factors, we could show that transcription factors bind to at least five specific enhancer sequences and mediate the activity of the IE1 gene enhancer in vitro (5).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17024-05 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

The Genetic Lesions of Tay-Sachs Disease

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

PI: R. Myerowitz Research Chemist LBM, NIDDK

Others: C. Costigan Special Volunteer LBM, NIDDK

L. Stewart Special Volunteer LBM, NIDDK

COOPERATING UNITS (if any)

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Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.2

PROFESSIONAL:

2.2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Tay-Sachs disease is an autosomal recessive disorder caused by mutations in the alpha-chain polypeptide of the A form of beta-hexosaminidase, a lysosomal enzyme composed of two chains (alpha,beta). Such lesions result in a spectrum of disease states ranging from severe to mild. Although the disorder is in general rare, Ashkenazi Jews, have a 10-fold higher gene frequency than the general population for a severe form of the disorder known as "classic" Tay-Sachs disease. It has been assumed that only one mutation gives rise to the severe form of Tay-Sachs disease in this ethnic group.

We previously found that Ashkenazi Jewish patients with classic Tay-Sachs disease lacked mRNA coding for the alpha-chain of beta-hexosaminidase, but retained a grossly intact alpha-chain gene indicating the presence of a subtle defect. During the past year we have completed sequence analysis of the promoter region, exon and splice junction regions and polyadenylation signal area of the mutant gene. Only one difference was observed between these sequences: at the 5' boundary of intron 12 a guanosine in the conserved splice junction dinucleotide sequence GT had been altered to a cytidine. The alteration is presumed to be functionally significant and to result in aberrant mRNA splicing.

We developed an assay which utilized the polymerase chain reaction to amplify the region encompassing the lesion to screen patients and carriers for the splice junction mutation. In two Ashkenazi patients tested only one allele harbored the splice junction mutation, others were negative for this mutation, and 30% of the obligate heterozygote carriers tested displayed the mutation. These results demonstrate that at least two lesions underlie the classic form of Tay-Sachs disease in the Ashkenazi Jewish population. We have isolated alpha-chain genomic clones from an Ashkenazi Jewish patient negative for the splice junction lesion and are sequencing critical region of this gene to determine this second mutation.



DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18000-23 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Hormone Dependent Development of Mammary Gland

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

PI: Y.J. Topper Scientist Emeritus LBM, NIDDK

Others: L. Sankaran Expert LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

This project was terminated as of September 30, 1987.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18007-09 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

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

PI: E.F. Grollman Medical Officer (Research) LBM, NIDDK

Others: S. Doi Visiting Fellow LBM, NIDDK
J.O. Joseph Summer Student LBM, NIDDK

COOPERATING UNITS (if any)

LBM, NIDDK (L.D. Kohn); NCI, DCBD (S. Shifrin); Univ. of Pennsylvania School of Medicine (N.J. Philp); USUHS (D. Fombaccini); Univ. of Pisa (C. Marcocci); M.I.T. (H.F. Lodish)

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Laboratory of Biochemistry and Metabolism

SECTION

Section on Cell Regulation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.5

PROFESSIONAL

2.25

OTHER

0.25

CHECK APPROPRIATE BOXES)

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

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

A primary role of the thyroid cell is to synthesize and secrete thyroid hormones, thyroxine and triiodothyronine. Taking into consideration the variable intake of iodide, this conversion process is accomplished in a regulated manner and involves numerous steps: concentrated uptake of iodide from the bloodstream and involves membrane of the cell; vectorial transport of iodide through the cell, efflux of iodide across the apical membrane into the lumen of the thyroid follicle; synthesis of thyroglobulin, vectorial transport of thyroglobulin to the follicular lumen; iodination of thyroglobulin; regulated storage of iodinated thyroglobulin; resorption into the cell; and degradation with reutilization of the iodine and secretion of thyroid hormones into the bloodstream. This process is further regulated by hormones including thyrotropin (TSH), adrenergic agents, insulin, cortisol, insulin-like growth factors, and iodine itself. These hormonal influences regulate the many steps described above and these processes have been the focus of some of our work over several years. My work continues to focus on transport proteins involved in the uptake as well as the efflux of iodide in the thyroid. My work characterizes the properties of this transport and the hormones and agents that influence, regulate or block these processes. A third direction we are pursuing is the iodinated intermediates between iodide and thyroid hormones mentioned above. This work includes the chemical modification of thyroglobulin as a means of understanding the role of hormone-rich iodopeptides in the synthesis and release of thyroid hormones from thyroglobulin. The work is being focussed at present on the thyroglobulin from patients with endemic goiter, a condition where in the absence of nutritional iodide, the thyroid compensates both in the size and function of the gland to provide the essential thyroid hormones. Over 400 million individuals worldwide are at risk of illness resulting from iodine deficiency. The work continues to investigate the role of ion fluxes as important early events in the action of hormones, as well as the complex pathway of iodine metabolism in the synthesis and release of thyroid hormones in normal function and pathology of the thyroid.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18008-22 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (60 characters or less. Title must fit on one line between the borders.)

Cell Regulation by Pharmacodynamic and Autoimmune Agents Acting on Cell Membranes

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

PI: L.D. Kohn Medical Director, USPHS, and LBM, NIDDK
 Chief, Section on Cell Regulation

Others: T. Akamizu Visiting Fellow LBM, NIDDK
 S. Aloj Visiting Scientist LBM, NIDDK
 J. Chan Guest Researcher LBM, NIDDK
 O. Isozaki Visiting Fellow LBM, NIDDK
 B. Shashikumar I.R.T.A. LBM, NIDDK
 K. Tahara Visiting Fellow LBM, NIDDK

COOPERATING UNITS (if any) NIDDK (E.F. Grollman, Vera Nikodem & S. Taylor); U. Pisa, Italy (A. Pinchera & C. Marccoci); U. Naples (R. DeLauro & E. Consiglio); U. Florence (R. Toccafondi & C.M. Rotella); Clin Endo, SP Brazil (G. Medeiros-Neto); NCI (S. Shifrin & W. McBride); CCHD (W. Gahl); NIDR (M. Lerman & A. Notkins); U. MD (W.A.

LAB/BRANCH Valente; Guy's Hosp., London (M. Sheppard)
 Laboratory of Biochemistry and Metabolism

SECTION

Section on Cell Regulation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

7.5

PROFESSIONAL

6.5

OTHER

1.0

CHECK APPROPRIATE BOXES)

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

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

Structure-function relationships in the mechanisms by which glycoprotein hormones (thyrotropin), autoantibodies, alpha 1-adrenergic agents, insulin, insulin-like growth factors (I and II), bacterial toxins (cholera and pertussis, for example), the anti-viral protective agent, interferon, and interleukins - alone and in combination - interact with and transmit their message through the cell membrane to affect cell function and pathology are being defined. Studies using monoclonal antibodies and the idiotype anti-idiotype theory explore the structure of the receptors for these ligands and the importance of these relationships to the expression of thyroid hyperfunction in Graves' disease; to organ-specific autoimmunity (Graves' disease, Hashimoto's disease, and diabetes); to fluid losses in intestinal diarrhetic states; to thyroid storm and the sympathetic overactivity syndrome of tetanus; to the ability of hormones to modulate the oncogenic state; and to the mechanism by which toxins subvert normal mechanisms to impose their pathological effects. Studies continue to evaluate the role of different signal transduction mechanisms - cAMP, Ca/phosphoinositide and arachidonate - by these agents for growth and differentiation for example, thyroglobulin biosynthesis, thyroglobulin biodegradation to T3 and T4, and the transport of T3, T4, monoiodotyrosine, diiodotyrosine, and other amino acids from the lysosome. The role of phosphate and carbohydrate moieties in thyroglobulin structure and post-translational processing is being studied. Studies explore lipid regulation of receptor expression, with special emphasis on neuronal and thyroid cell growth and development as well as the hormonal regulation of lipid metabolism, LDL receptor expression, and cholesterol biosynthesis. Studies to clone the TSH receptor and define its structure and regulatory control at a gene level are in progress as are studies to define the mechanisms by which TSH, insulin, IGF-I and other ligands regulate gene expression.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18009-09 LBM

PERIOD COVERED

January 1, 1988 through September 30, 1988

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

Endocytosis, Secretion and Compartmentalization in Mutant CHO Cells

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

PI: A.R. Robbins Research Geneticist LBM, NIDDK

Others: C.W. Hall Research Chemist LBM, NIDDK

S.M. Laurie Visiting Associate LBM, NIDDK

C.F. Roff Senior Staff Fellow LBM, NIDDK

COOPERATING UNITS (if any)

Department of Biochemistry, School of Public Health, Johns Hopkins University
(S.S. Krag)

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Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.3

PROFESSIONAL:

3.0

OTHER

0.3

CHECK APPROPRIATE BOX(IES)

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

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

This project transferred from GBB. The former project number was Z01 DK 52009-09 GBB.

Our approach to dissecting the processes of endocytosis, glycoprotein biosynthesis and sorting is through isolation and analysis of mutants. We have previously shown that most CHO cell endocytosis mutants fall into two genetic complementation groups, End1 and End2; both classes of mutants are defective in endosomal, but not lysosomal, acidification. Having identified a candidate for the End2 protein, Calvin F. Roff has developed a novel preparative three-dimensional gel procedure for purification of this (and other) membrane proteins in quantities sufficient for immunization.

To obtain new classes of mutants we devised an isolation procedure for cells defective in lysosomal acidification. Exploiting the quenching of fluorescein at acidic pH we screened for cells exhibiting above normal fluorescence after pulse-chase labeling with fluoresceinated dextran (Mr 70,000). One such mutant accumulates dextran in large non-acidic vacuoles; based on functional assays, its endosomal acidification is unimpaired.

Susan M. Laurie has continued analysis of LEFIC, a mutant Ltk- cell which is cross-resistant to toxins but has normal endosomal function. The principal defect in LEFIC appears to involve movement of membrane proteins from late Golgi regions to the plasma membrane. Oddly, delivery of membrane proteins in LEFIC is more severely affected than is secretion of soluble proteins.

To further characterize mutants defective in early steps in the pathway of N-linked glycosylation, Clara W. Hall has developed an *in vitro* system for biosynthesis, translocation and elongation of lipid-linked oligosaccharides in intact microsomal vesicles. Conditions for measurement of translocation of lipid-linked Man5GlcNAc2 (the intermediate believed to move from external to luminal faces of the ER) without elongation of the oligosaccharide have been established.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 18010-01 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

The Role of Intracellular Traffic in HIV Infection

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

PI: J.A. Hanover Senior Staff Fellow LBM, NIDDK

Others: C. Starr I.R.T.A. LBM, NIDDK
B. Wolff Visiting Associate LBM, NIDDK

COOPERATING UNITS (if any)

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Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.5

PROFESSIONAL

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

An investigation of CD4, the presumed T-cell receptor for the human immunodeficiency virus, has shown that surface expression of this protein is blocked by tunicamycin, a potent inhibitor of glycosylation, under conditions where alternate surface receptors are unaffected. Initial studies employing acute lymphoblastic leukemic cells have been extended by the successful transfection of a plasmid containing the cDNA for CD4 into Chinese hamster ovary cells. Subsequent cotransfection with another plasmid containing the multiple drug resistant gene, has permitted the isolation of stable clones expressing large amounts of CD4. Preliminary data on this material indicates the presence on CD4 of complex or multi-antennary hybrid oligosaccharides.

A targeting sequence has been identified which is sufficient to allow protein molecules as large as 0.5×10^6 to pass through the nuclear envelope. This has been assessed in our laboratory by chemically coupling peptides having the nuclear localization sequence to a highly fluorescent cytoplasmic protein. The sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Cys is sufficient to target these conjugates to the nucleus. The tat and art gene products of HIV have sequences very similar to this targeting domain. The approach we have taken is to chemically synthesize peptides corresponding to the tat sequence examined the ability of this peptide to confer nuclear localization. In addition to these studies, we have begun to examine the structure of the nuclear pore complex, across which the many nucleocytoplasmic exchange processes must occur. The structure of the pore complex is explored using recombinant DNA techniques. The major nuclear pore glycoprotein np62 has been cloned and a large segment sequenced.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18011-01 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Cell Specific Activity of Elements within the HIV-LTR

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

PI: L. Hennighausen Visiting Associate LBM, NIDDK

Others: P.A. Furth Special Volunteer LBM, NIDDK
 P. Ghazal Visiting Fellow LBM, NIDDK
 H. Lubon Visiting Associate LBM, NIDDK

COOPERATING UNITS (if any)

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SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2

PROFESSIONAL

2

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

Work in this laboratory had revealed information about promoter, enhancer and repressor elements that regulate human cytomegalovirus (HCMV) genes and the relationship between gene expression and binding of transcription factors to cis-acting elements. In extension of this work, we have examined transcriptional regulation from the HIV-1 long terminal repeat (LTR) which contains control elements required for viral activation. *In vitro* transcription systems were established from lymphoid and nonlymphoid cell lines and accurate initiation of transcription from the HIV LTR was observed. New findings show that the *in vitro* systems can direct enhancer-dependent transcription from the HIV-1 LTR in several cell types, including T-cells, B-cells and epithelial cells (1). Although regulatory elements within the enhancer core sequences are preferentially active in lymphoid cells, only a limited host cell restriction of HIV transcription initiation *in vitro* was observed (1). The possibility of transcription factors binding to the enhancer sequences and mediating transcriptional stimulation was investigated. Using sensitive binding and competition assays it was shown that proteins bind to the HIV-1 enhancer core sequences (1) and that protein-DNA interaction is necessary for enhancer dependent transcriptional stimulation (2).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18012-04 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Direct Measurement of Forces between Membranes or Macromolecules

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

PIs:	V.A. Parsegian	Guest Worker	LBM, NIDDK
	D.C. Rau	Expert	LBM, NIDDK
Others:	R. Podgornik	Visiting Fellow	LBM, NIDDK

COOPERATING UNITS (if any)

Brock Univ., Ontario (R.P. Rand); Univ. British Columbia, Vancouver (E.A. Evans); Univ. Minnesota (D.F. Evans)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

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

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

This project transferred from LCB. The former project number was Z01 DK 25047-03 LCB.

The theme of this project is that actual measurement of forces between large molecules will teach us how these molecules interact to form the functioning units of a living cell. The results of these direct measurements are quite different from earlier expectation. For example, we have learned that in the important last few nanometers where molecules approach contact, interaction is dominated by a continuously varying work of removal of water solvent from their surfaces. Between phospholipid bilayer membranes, these important "hydration forces" are exquisitely sensitive to very small changes in the composition of the lipid polar groups, changes that are under the biochemical control after formation of the membrane structure.

We have recently succeeded in measuring not only the force vs. separation of molecules but also the change in their thermal motion during mutual approach. Outside the 1 nanometer range of direct hydration repulsion, it appears that macromolecules move or undulate to repel by the "steric" action of molecules colliding. However these collisions never involve molecular contact but rather occur through long-range forces between molecular surfaces. The result is a form of interaction qualitatively different from any that has been assumed to be responsible for molecular assembly.

One can now use these data to see how the measured forces act at the functional level of, say, controlling the contact and fusion of membranes as in a secretory process, or determining the packing of DNA or other long molecules, or perturbing the rearrangements of protein structure that effect the "gating" of trans-membrane ionic channels, or even affect the activity of enzymes whose function depends on particular forms of packing components. We have been carrying out work on each of these processes.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18013-01 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Physics of Ionic Channels and other Proteins with Aqueous Cavities

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

PI: V.A. Parsegian Guest Worker LBM, NIDDK

Others: E. Curtin Special Volunteer LBM, NIDDK

J.J. Kasianowicz Special Volunteer LBM, NIDDK

S. Leshner Special Volunteer LBM, NIDDK

J. Zimmerberg Guest Worker LBM, NIDDK

COOPERATING UNITS (if any)

Office of Naval Research; Johns Hopkins University (Dr. Andrew Harris); Ohio State University (Dr. Ann'Walter); UCLA and Marine Biology Lab, Woods Hole (Dr. F. Bezanilla)

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2 2/3

PROFESSIONAL

1 2/3

OTHER

1

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

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

The theme of this work is that proteins, especially those designed to transport ions across channels, are sensitive to osmotic stress from materials unable to enter their aqueous cavities. We have used the ability to exert osmotic stress to measure the amount of water that goes in or comes out when a protein, particularly a trans-membrane ionic channel opens or closes.

Among our accomplishments during the past year, we have determined that some 20 to 40 water molecules enter a typical potassium channel of the squid giant axon when it opens under electrical stimulation. These measurements, on whole axon preparations, suggest that rather significant changes in protein structure must occur during channel opening. Such large changes are quite different from what is usually imagined to be channel "gating".

We have developed a new method for separating out vesicles containing single channels and for incorporating these into artificial systems. This development is enabling more controlled protein reconstitution into artificial bilayer membranes for single-channel study.

Major effort has been successfully expended in designing computer hardware and software to allow high-speed data acquisition while recording channel electrical activity, concurrently analyzing results and putting the variation of experimental conditions under computer control. This system, a prototype for personal computer laboratory use, also makes possible better connection with powerful mainframe equipment for more sophisticated data analyses if need be.

Finally we have begun to look at the sensitivity of enzyme activity to osmotic stress to see how such stress, naturally occurring in living cells, is a part of intracellular activity.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18014-04 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Structure and Physical Properties of DNA and DNA-Protein Complexes

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

PI: D.C. Rau Expert LBM, NIDDK

COOPERATING UNITS (if any)

George Mason University, Fairfax, VA (Dr. H. Chen); LMB, NIDDK (Dr. J. Nickol); LCP, NIDDK (Drs. M. Riehm and E. Charney); University of Nevada, Reno, Nevada (Dr. R. Harrington)

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TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

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

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

This project transferred from LCB. The former project number was Z01 DK 25050-03 LCB.

Major emphasis has been placed on identifying and overcoming the problems associated with photochemical electric dichroism. This technique is a hybrid of classical electric dichroism and the methodologies developed by molecular biologists for examining DNA-protein complexes. The end result will be a technique that can sensitively and selectively map the topology of DNA folding or wrapping in specific DNA-protein complexes of biological importance. The problems we have encountered primarily result from the very large electric fields applied across the sample and the very intense UV laser light that must be employed. Electrode poisoning is the primary effect. A greater emphasis on electrode cleaning and preparation appear essential for optimal results. Additionally, buffer conditions for the dichroism experiment are far more critical for this type of experiment than classical dichroism because of non specific background nicking of DNA.

Improvements in signal quality and differences observed in photodamage probability between oriented and unoriented reconstituted nucleosomes indicate that the technique will work and become a dominant structural technique for protein-DNA complexes with sensitivities and specificities comparable to footprinting.

We have installed a new computer-linked data collection system to our electric birefringence and dichroism apparatuses. The LeCroy system gives a greater time resolution and makes data analysis faster and more convenient. Work on other projects can now proceed at a faster rate. We have expanded our work on bent DNA fragments to include oligo A tract sequences that show interesting flanking sequence properties. Initial results on bipolar myosin filaments indicate a great deal of internal motion and that this motion is different for phosphorylated and unphosphorylated myosin. Building on our previous results we are about ready to construct dynamical models for the bipolar filament.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18015-04 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Histamine Release from Beige Mouse Mast Cells

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

PI: J. Zimmerberg Guest Worker LBM, NIDDK

Others: M.J. Curran Staff Fellow LBM, NIDDK

COOPERATING UNITS (if any)

Rush Medical College, Chicago, IL (Fredric S. Cohen, Ph.D.)

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TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

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

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

This project transferred from LCB. The former project number was Z01 DK 25053-03 LCB.

As a consequence of fusion of an intracellular granule with the plasma membrane, an exocytotic pore (which characterizes the omega-figure seen in electron micrographs of secreting cells) connects the granule interior with the extracellular medium. With the use of the patch clamp technique and mast cells from beige mice, we have discovered small fusion pores which initiate exocytosis. These pores are much smaller than the limit of electron microscopy. Our techniques allow us to follow the rapid enlargement of this pore for every fused granule. Large, stepwise increases in capacitance are recorded that result from the fusion of large, individual secretory granules to the plasma membrane. Pores sizes are derived from the impedance analysis of the equivalent circuit.

An analysis of hundreds of such fusion events show rich and varied kinetics, suggestive of a dynamic fusion pore structure. The widening can be rapid or slow, monotonic or fluctuating. Thus the exocytotic pore has fundamental differences from stable membrane channels and gap junctions, and quick, fluid lipid fusion. We envision a protein/lipid complex.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18016-01 LBM

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders)

Cell-Cell Fusion due to Influenza Hemagglutinin

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

PI: J. Zimmerberg Guest Worker LBM, NIDDK

COOPERATING UNITS (if any)

Laboratory of Theoretical Biology, NCI (S.J. Morris, Ph.D.;, R. Blumenthal, Ph.D.)

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TOTAL MAN-YEARS

1

PROFESSIONAL:

1

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

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

Initial events in infection by enveloped viruses, such as influenza, rabies, herpes and HIV, involve binding of viruses to host cell plasma membranes followed by fusion to the plasma membrane or internalized membrane after receptor-mediated endocytosis. We are using a recently developed series of real-time fluorescence probe methods to follow the fusion of viruses to cultured cells or human red blood cells (RBC) to cells expressing viral spike glycoproteins (SGCs) on their extraplasmic surface. Our results of kinetic analysis of these interactions suggest that the membrane rearrangements of proteins and lipids necessary for fusion first allow lipid exchange followed rapidly by establishment of one or more fusion pores, which allow exchange of soluble molecules. We directly image the fluorescent dyes by image enhanced video light microscopy: one can analyze the spatial redistribution of the fluorescent probe between the fusing cells. We developed methods using low light image enhanced video microscopy of live cells to analyze a series of objectives concerned with early events in viral protein-mediated membrane fusion. Our hypothesis is that fusion is established by the viral fusion protein forming a pore which gates the passage of molecules between the RBC and target cell according to size and charge. We hope to establish a molecular "time table" which correlates the movement of membrane and cytoplasmic molecules during the fusion process. Using a combination of patch clamp and videomicroscopy, we can also correlate membrane and core probe movements with pore formation.

ANNUAL REPORT OF THE LABORATORY OF CHEMISTRY

NATIONAL INSTITUTE OF DIABETES, DIGESTIVE AND KIDNEY DISEASES

SECTION ON BIOCHEMICAL MECHANISMS

CHEMISTRY OF IMIDAZOLES AND BIOIMIDAZOLES

In various sections of this report, we describe significant and valuable applications of histidine analogues in biochemical and pharmacological studies. Such studies could have been performed many years ago, but for the fact that these analogues had not been available through classical or obvious synthetic routes. Even methods suitable for simple imidazoles may not be applicable to complex bioimidazoles, because of the additional functional groups and chirality. Thus, nonclassical methods (e.g., photochemical radical substitution, one-electron reduction, etc.) were developed to fit these gaps.

Even more novel methods are always being sought to provide analogues still inaccessible. We have now developed procedures for the conversion of aminohistidines into azido and nitrohistidine, of amino to chloro, bromo and iodo, of trifluoromethyl into methyl, cyano, carboxy, carbomethoxy, etc. Recently, we synthesized 2- and 4-(pentafluoroethyl)-histidines by photochemical radical substitution. These compounds are converted by base into the corresponding (trifluoroacetyl)-histidines, which have such reactive carbonyl groups that they may serve as affinity labels for histidine-binding sites. The trifluoroacetyl-imidazoles can be reduced to the secondary alcohols, also obtainable by direct condensation of imidazoles with trifluoroacetaldehyde. In turn, the secondary alcohols can be oxidized to the trifluoroacetyl ketones. Upon treatment with methanolic base, (trifluoromethyl)-histidine can be converted into (trimethoxymethyl)-histidine and pentafluoroethyl into the corresponding ketal. These ortho functionalities are also of interest as potential covalent affinity labels.

Ring-trifluoromethylated imidazoles show the unique property of losing hydrogen fluoride above pH 8 to form metastable difluorodiazulvenes, which then react with any available nucleophile to form new covalent bonds. Such intermediates, derived from trifluoromethylhistamine or histidine, may be able to serve as covalent affinity labels for specific binding sites, both *in vitro* and *in vivo*. It would be desirable, therefore, to have available a series of trifluoromethyl analogues with a range of reactivities, and to be able to correlate reactivity with some substituent parameter. Our discovery of a simple photochemical method for the trifluoromethylation of imidazoles has made available a large series of analogues for study. We have found that the reactivities of some members of the group can be correlated with the special electronic effects of certain substituents (capable of hyperconjugation or back-bonding). Computer analysis of reactivity data for a series of trifluoromethylimidazoles has provided a linear free energy relationship in which $\log k_r$ correlates with both inductive and resonance components of the respective substituents. According to computer-based predictions, the fluoro group would provide the ideal combination of acidity and reactivity under physiological conditions. We have, therefore, developed procedures for sequential photochemical introduction of fluorine and trifluoromethyl into imidazoles and have verified the predicted reactivities. We are now involved in the preparation of peptide hormones containing these substituents. Photochemical introduction of the trifluoromethyl group has been found practical for more complex imidazoles and studies are under way for the synthesis of the trifluoromethyl analogue of the anti-ulcer drug, cimetidine.

A number of 4-X-bioimidazoles are accessible by direct electrophilic substitution (nitro, halo); 2-X-bioimidazoles are far less accessible and can be obtained only by indirect and, often, very tortuous routes. Prior to our efforts in this area, the majority of 2-X-bioimidazoles were totally unknown. By far, the simplest 2-substituted bioimidazole now obtainable is the 2-iodo compound. 2-Iodo-L-histidine and 2-iodohistamine, unknown prior to 1985, are now accessible in large quantity by reduction of the corresponding 2,4-diiodo compounds in refluxing hydrochloric acid. Recent work has revealed, to our great surprise, that the same reductions can occur in hot water alone! Since 2,4-diiodo-5-methylimidazole does not show such behavior, we infer that the side-chain amino group plays the remarkable role of intra-molecular, regiospecific reducing agent. 2-Iodohistidine is of interest not only because of its potent antimalarial activity (see later), but because it may serve as a valuable intermediate for the synthesis of other 2-X-histidines. We are currently exploring several schemes for such conversions.

ANTIMALARIALS

Our development, in 1971, of a photochemical route to ring-fluorinated aromatics and heteroaromatics has led to the synthesis of a wide variety of fluoro analogues of imidazole-based metabolites. Many of these compounds have shown interesting properties as agonists or antagonists and have proved useful as research tools and as possible chemotherapeutic agents. A striking difference has been found between 2-fluoro-L-histidine (2-FHIS) and the 4-fluoro isomer. While the former compound is readily incorporated into new protein in place of histidine (both in bacteria and mammals), the 4-fluoro isomer is not incorporated at all. Furthermore, 2-FHIS shows antibacterial, antiviral, antileukemic and antimalarial properties; again, the 4-fluoro isomer shows none of these activities. From our ^{13}C NMR studies of other substituted histidines, we now suspect this differentiation to be based on tautomer preference in the imidazole ring; thus, 2-X-histidines resemble histidine in preferring the 1,4-tautomer, while 4-X and 2,4-di-X-histidines prefer the unnatural 1,5-tautomer. We found such differentiation in a variety of isomer pairs, including X = fluoro, iodo, trifluoromethyl. Furthermore, 2-fluorohistamine was found to be a potent agonist at the histamine H-1 receptor, while 4-fluorohistamine is active at the H-2 receptor. Again, tautomer preference may be the basis for such differentiation and may provide a rationale for the design of other agonists and antagonists.

We have become particularly interested in the antimalarial properties of 2-FHIS, since the compound is uniquely and selectively active against Plasmodium falciparum, that parasite which is notoriously resistant to chemotherapy. The organism has the unusual property of inducing production, within an invaded erythrocyte, of a protein containing as much as 70% histidine. The protein is found in "knobs" which are seen on the erythrocyte surface; these knobs are responsible for a very strong adherence of the infected erythrocytes to capillary endothelium, thereby sequestering parasitized cells which would normally be destroyed during passage through the spleen.

In cultures of infected erythrocytes, low concentrations of 2-FHIS not only inhibit cytoadherence but prevent maturation of the parasite and the appearance of knobs entirely. The assumption that these antiparasitic properties are due to the incorporation of 2-FHIS into the histidine-rich protein is probably unwarranted, since the treated parasite shows a general decrease in protein synthesis and rather low incorporation of ^3H -2-FHIS. As one of several hypotheses for the mechanism of action, we propose that 2-FHIS interferes with histidine as a promoter of the transport of some other amino acids into the cell. This hypothesis is supported by our earlier findings that 2-FHIS inhibits protein synthesis in cell and organ cultures but not in cell-free systems. Studies are in progress on the effect of 2-FHIS on facilitated amino acid transport. Unfortunately, the high antimalarial activity shown by 2-FHIS in vitro could not be extended, because the compound proved too toxic in monkeys. Such toxicity is surprising, since mice tolerate

as much as 500 mg/kg. We are now exploring metabolically stable derivatives of the amino acid analogue, in the hope of reducing toxicity. A large number of other substituted histidines have been screened for *in vitro* activity: 2-iodo showed good activity while 2-azido showed moderate activity. Surprisingly, the 2-chloro and 2-bromo analogues were inactive.

Laboratory-scale production of these histidine analogues is extremely time-consuming, involves multiple low-yield steps, and is limited to small batch operation. Our recent efforts to find alternative, and more economical routes have been successful - at least for 2-IHIS. Readily available 2,4-diiodo-L-histidine can be converted into mixtures of 2-IHIS, 4-IHIS and HIS by photoreduction, catalytic hydrogenation or reduction with titanium trichloride. The last method is especially promising, providing yields of 2-IHIS up to 20% in this one-step process. More recently, we have found that 2,4-diiodo-L-histidine can be reduced selectively with hot 3N HCl to 2-iodo-L-histidine, without formation of any of the 4-iodo isomer. Despite its promise in *in vitro* tests, the 2-iodo analog proved inactive in monkeys (but also nontoxic). It is likely that mammals possess a metabolic pathway for deiodination of the iodo compound. Deiodination of 4-iodohistidine in rats had been observed previously. Preliminary *in vitro* studies show that 2-iodohistidine is much less effective than 2-FHIS in interfering with protein synthesis and, thus, appears to operate by a different mechanism. We postulate that the iodo compound may be blocking nutrient diffusion holes in the erythrocyte membrane. The absence of an involvement in active transport channels is supported by our observation that 2-iodohistamine and its α -N derivatives also show some *in vitro* inhibitory activity. The role of the iodine atom may be steric or lipophilic (and probably not electronic). In order to attempt any structure-activity correlation, data is needed for other substituted histidines with large, lipophilic groups at C-2 (iPr, tBu, Ph, Bz, etc.). We are now developing new synthetic methods to obtain such compounds, based on (1) cyclization of dibenzoylaminoethylenes with acyl halides, (2) synthesis and hydrogenolysis of ketones and (3) imidazole-ring substitution with photochemically generated radicals. Strong efforts to develop method (1) have not been promising; method (2) has only limited applicability; preliminary studies with method (3) are very encouraging. Further clues to the design of effective antimalarials may be achieved from knowledge of the mechanisms of action of these histidine analogues. To this end, a synthesis of 14 C-2-fluorohistidine has been developed. We have also demonstrated that H-4 in 2-iodohistidine can be exchanged with isotopic hydrogen under alkaline conditions.

HYPOXIC CELL SENSITIZERS

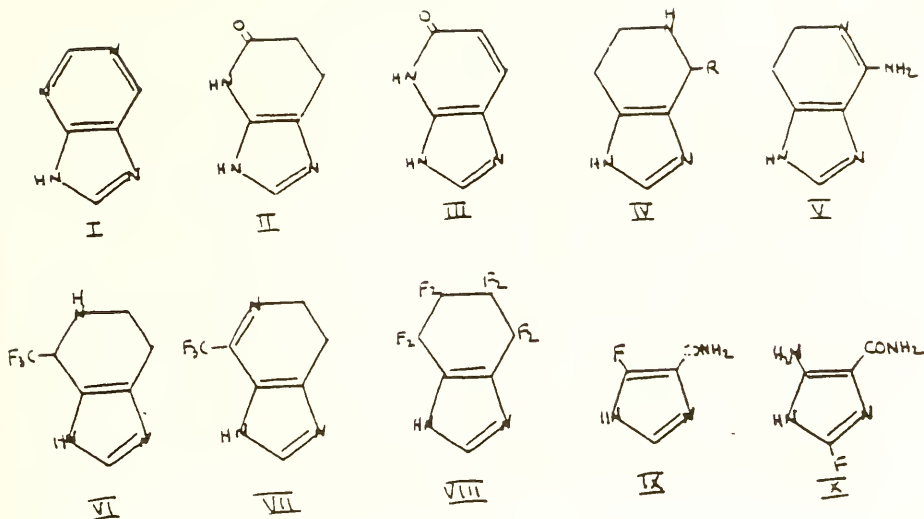
The valuable properties of nitroimidazoles as radiation sensitizers and as selective cytotoxic agents for cancer treatment have stimulated considerable research into mechanisms of action and metabolic fate of the drugs. We have proposed three theories for the mechanism of action: (1) Thiols are known to add to the 4,5-double bond of nitroimidazoles and, thus, such compounds may interfere with normal cellular functions by binding cysteine, glutathione, SH enzymes, etc. (2) Nitroimidazoles may be reduced, *in vivo*, to hydroxylaminoimidazoles which can function as supernucleophiles in cleaving the phosphate ester bonds of polynucleotides; unfortunately, synthetic hydroxylaminoimidazoles have been found so unstable that their potential as nucleophiles cannot be investigated. As an alternative, we are devising synthetic methods for hydrazinoimidazoles; these compounds should be significantly more stable than hydroxylaminoimidazoles and yet, should possess the same nucleophilic power inherent in hydroxylamine functions. Our primary interest is in 2-hydrazinoimidazoles: efforts to prepare these compounds by displacement of fluorine in 2-fluoroimidazoles have been unsuccessful; we are now trying reduction of 2-diazonium imidazoles with borohydride-metal combination. (3) Reduction of the nitro group by nonnucleophilic agents leads to nitro radicals; we believe these heterocyclic radicals capable of alkylating cell constituents and interfering with metabolism. To this end, we are now studying the anaerobic reduction of nitroimidazoles with one-electron transfer agents (e.g., titanous

chloride). A critical factor in the development of new sensitizers is the availability of a practical synthesis of 2-nitroimidazoles. Although we have already published several routes, they are not truly practical and cannot be used with polyfunctional bioimidazoles. We are currently exploring methods involving nonselective radical nitration with photochemically generated nitro radical.

Misonidazole is an alkylated 2-nitroimidazole which has been found quite effective in sensitizing cancer cells to radiation and in reducing the radiation dose needed to effect significant cell destruction. Unfortunately, the compound has to be used at such high levels as to produce serious side effects and may not be released by FFA. We have postulated that the introduction of nitro groups into more natural imidazoles (histamine, histidine, etc.) may produce the desired alien molecule. Indeed, several such compounds have shown *in vitro* activity comparable to that of misonidazole. Evaluation of the clinical effectiveness of this series of compounds in animals is in progress.

IMIDAZOLE ANTIVIRALS AND OTHER MEDICINALS

The notable success of virazole and deazapurine systems as antivirals has stimulated research into further modifications of the purine (I) ring system, especially those involving replacement of ring nitrogen with carbon. Analogues synthesized to date have required laborious multistep processes and have given only low yields. We have devised a number of simple syntheses which produce deazapurine analogues in good yield and with few steps. Reduction of 4-nitrohistidine ester or of 4-nitroimidazolepropionic ester leads to II. Reduction of 4-nitrouroacnic ester gives the stable 4-aminouroacnic ester, but subsequent irradiation converts the *trans* olefin to *cis* and the product cyclizes to III. Condensation of histamine with aldehydes gives series IV and cyclization of 4-(trifluoromethyl)-histamine with ammonia gives V. Series VI is obtained by condensation of histamine with trifluoroacetaldehyde, VII by cyclization of 4-(trifluoroethyl)-histamine with base, and VIII by photochemical reaction of imidazole with 1,4-diodo-perfluorobutane. Compounds which we have previously found to have significant antiviral activity (IX and X) are also being modified somewhat. Systems III-VII can be dehydrogenated to the fully aromatic systems with selenium dioxide. These compounds, with or without ribose attachment, will be evaluated for antiviral activity, particularly against AIDS.



A major program has been initiated to devise synthetic routes to hydrazinoimidazoles, which we consider promising antimetabolic agents. Such compounds are still unknown and predictions of stability and chemistry are based on the properties of phenylhydrazines. Thus far, efforts to replace fluorine in 2-fluoroimidazoles with hydrazine or to reduce 2-diazoniumimidazoles have failed. A number of alternative approaches are still open and are being explored.

TRH ANALOGS

The simple tripeptide, L-pyroglutamyl-L-proline amide (TRH), exerts marked cardiovascular, behavioral and analeptic effects, through activation of the sympathoadrenomedullary system. These effects appear to be unrelated to its action on the hypothalamo-pituitary axis to release thyrotropin and prolactin. Involvement of TRH in many nonendocrine functions of brain is also suggested by its distribution and the presence of high affinity binding sites outside the hypothalamus and pituitary. TRH has shown promise in the treatment of various forms of shock, as an analeptic, antidepressant and in promoting the regeneration of injured spinal cord. Practical clinical utility of the peptide is limited, however, by this very multiplicity of biological activities, as well as by its very low biological half life. The presence of degrading enzymes in blood serum, a difficulty in crossing the blood-brain barrier because of its polar structure, and the unavailability of facilitated or receptor-mediated transport - all serve to limit severely the survival of exogenous TRH and its delivery to the brain. On the other hand, the multiplicity of significant (or even vital) physiological activities of TRH argues strongly for the search for synthetic analogues which can not only overcome these limitations of stability and penetration but also achieve separation of the various activities.

The synthetic analogues used in our previous and current studies have all involved modification (or replacement) of the imidazole ring of histidine; these analogues have produced dramatic dissociation of some activities, suggesting that the different physiological functions of TRH may be mediated through different receptors or subtypes thereof. In contrast to TRH, 4-F-Im-TRH and 2-CF₃-Im-TRH do not bind to pituitary GH4 cells *in vitro* nor stimulate prolactin release from them; such results would immediately suggest the analogues to be nonfunctional. On the other hand, systemic injection or direct microinjection into rat brain of either analogue not only results in increased cardiovascular (CVS) effects (heart rate, blood pressure) comparable to those found with TRH, but also in release of prolactin at 2-3 times the level observed with TRH. In the whole animal, therefore, prolactin release can be controlled from receptor sites *outside* the pituitary. Enhanced CVS activity is also evident in 4-CF₃-Im-TRH and 4-NO₂-Im-TRH, and it would seem that the receptor for CVS activity is essentially indifferent to the position, size or nature of the imidazole ring substituent. The fallacy of this conclusion is demonstrated by the greatly reduced activity of 4-I-Im-TRH and the total inactivity of 2,4-I₂-Im-TRH. Furthermore, replacement of histidine by an aliphatic amino acid (e.g., norvaline) also results in the loss of CVS activity. The spectrum of CVS and other activities are summarized in Table I.

Physiological Activities of TRH Analogues ^a

Compound ^b	CVS Activity	Prolactin Release	TSH Release ^c	CNS Activity ^c
TRH	+++	+	+++	+++
4-F-TRH	++	++ ^d		
4-CF ₃ -TRH	+++	++		
2-CF ₃ -TRH	+++	+++		
4-I-TRH	0(+)	+ ^e	0	
2,4-I ₂ -TRH	0	+	0	
4-NO ₂ -TRH	+++	0	0	
Nva ² -TRH	0	+	0	+++ ^f

^a By intra-arterial administration in conscious rats, unless otherwise indicated.

^b Substitutions are all on imidazole ring of histidine.

^c Reported in the literature.

^d Both CVS and prolactin release are observed following central administration.

^e Active at higher dose (30 μmol/kg).

^f Ten times more potent than TRH in analeptic activity test.

It is evident from the Table that the structural requirements for CVS activity differ markedly from those for prolactin release and that, for the latter an imidazole ring may not be necessary at all. Equally striking is the evidence that structure-activity factors for the release of thyrotropin-stimulating hormone (TSH) do not parallel those for prolactin release. Although data is still being assembled, it is already apparent that CNS activity will show its own unique structure dependence.

It is now quite clear that at least four of the biological activities of TRH involve uniquely different receptors and that, after decades of effort in various laboratories, the separation of these activities has at last been achieved. Thus, 4-NO₂-TRH, highly selective for CVS activity, may be useful in the treatment of various forms of shock without a concomitant enhancement of thyroid activity or of prolactin release. On the other hand, 2,4-I₂-Im-TRH or Nva²-TRH may be useful as diagnostic tools for the assessment of pituitary function without the risk of increased blood pressure and tachycardia induced by TRH. The iodinated analogue is particularly useful since it can be prepared readily with radioactive iodine. Furthermore, each of these selective agonists should provide a useful research tool for the study of the role and mechanism of TRH involvement in the respective function.

Receptor binding studies have now been completed for ten analogues of TRH, using rat pituitary, hypothalamus, brainstem and cortex tissues. In contrast to TRH itself, which binds in the nanomolar range, eight analogues bind in the micromolar range and two (4-NO₂-Im-TRH and 4-CF₃-Im-TRH) do not bind measurably to any of the tissues. Nevertheless, both these analogues show potent cardiovascular activity! Since binding assays are based on displacement of radiolabeled 3-Me-His²-TRH, we must consider the possibility that TRH binds to a class of receptors to which the 3-methyl analogue does not bind. Accordingly, binding studies are being repeated with labeled TRH itself. It is also possible that our analogues should not be viewed as TRH peptides at all, but as synthetic receptor ligands of a separate class. Finally, we may consider that these analogues operate by secondary interaction with other neurotransmitters. From our ¹³C NMR studies of tautomer preference in substituted histidines, we may conclude that 4-X-Im-TRH species will exist as the 1,5-tautomer and 2-X-Im-TRH species as the 1,4-tautomer (as with TRH itself). Indeed, we find that 2-CF₃-Im-TRH binds 100-fold more strongly than 4-CF₃-Im-TRH and it is clear that additional 2-X-Im-TRH analogues need to be examined. Unfortunately, these compounds are far more difficult to synthesize than the 4-X isomers, but a concerted effort has been initiated to obtain them.

Strong conclusions about structure-activity correlation are not yet possible. We have theorized that the imidazole ring of histidine is necessary for the CVS activity of TRH but is not essential for prolactin-releasing activity. The unexpected loss of CVS activity in 2,4-I₂-Im-TRH may be due to steric hindrance to binding at the TRH receptor. In addition to size, ring substituents vary in electronegativity, polarity, hydrophobicity and ability to participate in intra- and/or intermolecular hydrogen bonding. One or more of these variables may stabilize the physiologically relevant conformations of TRH, interfere with binding or promote binding to a specific receptor. In addition, a given substituent may stabilize either the π or τ tautomer of imidazole in histidine.

A number of other new imidazole-modified analogues of TRH have already been prepared and others are in progress. With data on the pharmacology and neurobiology of all these analogues, we hope to identify the structural requirements and limitations for each type of activity, as well as the role of imidazole pK, aromaticity and hydrophobicity. In order to determine whether both ring nitrogens are necessary for activity and whether imidazole tautomers can be differentiated, we are currently preparing analogues of TRH with other heterocyclic rings in place of imidazole. Receptor-specific analogues will also be prepared with increased resistance to enzymic degradation and more lipophilic prodrugs are planned to accelerate penetration to the brain.

CHEMISTRY, BIOCHEMISTRY AND PHARMACOLOGY OF BIOINDOLE ANALOGS

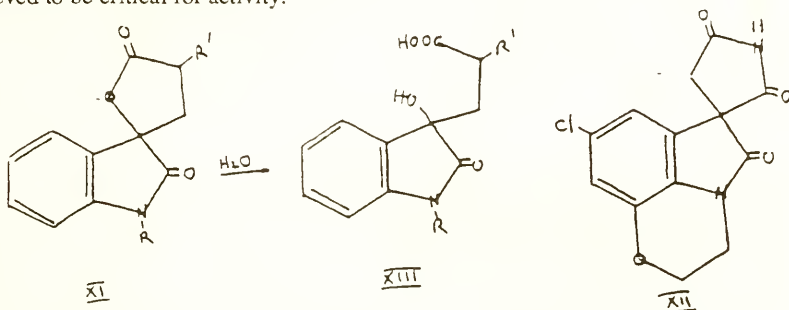
Tryptophan is an essential amino acid, serving as the precursor of the neurotransmitter, serotonin, and of the hormone, melatonin, in addition to its roles in enzymes and in receptor proteins. Tryptophan is metabolized in mammals by a pyrroloxygenase in the liver, where it can serve as a precursor of nicotinamide (Vitamin B) in some animals. In other tissues, tryptophan and related indoles are metabolized by a distinct oxygenase, the activity of which is dramatically increased (up to 100-fold) upon administration of bacterial lipopolysaccharides or interferon. The role of the oxygenase in the response of the organism to infection is unknown, however. We anticipated that certain 2-substituted tryptophans might serve as selective "suicide substrates" for these oxygenases. Analogs of tryptophan with electronegative substituents at C-2 had not been previously prepared. We have obtained 2-chloro and 2-bromo-L-tryptophan by radical halogenation, 2-trifluoromethyl-L-tryptophan by photochemical substitution, and 2-nitro-L-tryptophan as a minor product of direct nitration. Both the trifluoromethyl and nitro groups can be converted readily into other functions; some of these derivatives are of potential value as affinity and photoaffinity labels, as antibacterial agents and as photosensitizers in radiation therapy. 5-Azido-L-tryptophan has already been found effective as a photoaffinity label for tryptophan synthase.

The mechanisms of hydrolysis of the 2-halotryptophans at low pH have now been fully elucidated and reveal the involvement of intramolecular proton transfer in the conversion of the stable indole to the labile indolenine tautomer. An enzyme carboxyl group should also promote indolenine formation, suggesting the indolenine to be the true substrate for certain tryptophan enzymes. The first conclusive support for this concept is found in the demonstration that 2,3-dihydro-L-tryptophan and oxindolyl-L-alanine, analogs of the indolenine tautomer of tryptophan (tetrahedral carbon at C-3), are potent competitive inhibitors of tryptophan synthase and tryptophanase. Furthermore, the two enzymes show opposing specificity for the C-3 diastereoisomers of 2,3-dihydro-L-tryptophan suggesting that these enzymes catalyze their reactions via enantiomeric indolenine intermediates. The chiral center at C-3 in oxindolyl-L-alanine racemizes too readily to permit a study of opposing enzyme specificity. We have recently prepared the stable diastereoisomers of 3-hydroxy-oxindolyl-L-alanine (XIII) and, indeed, find the same opposing specificity for tryptophan enzymes as with dihydrotryptophan. The diastereoisomers of XIII have been identified by comparison with chiral synthons of the indole alkaloid, tryptoquivaline, whose absolute stereochemistry has been determined by x-ray crystallography. The inhibitory activity of XIII(R) on tryptophanase matches that of (3R)-2,3-dihydro-L-tryptophan. The lactone ring of XI is significantly strained and can be opened at C-3 by nucleophilic displacement. Thus, the 3-cyano and 3-azido analogs of XIII have now been prepared for study as inhibitors. In particular, the 3-azido analog may serve as a photoaffinity label. Reaction of XIII with DAST (diethylaminosulfur trifluoride) converts the 3-hydroxyl group into fluorine. Unfortunately, the fluorine atom appears to be far more reactive than anticipated, and recyclization to XI occurs almost without provocation.

The diastereoisomers of XI ($R = H$, $R' = \text{NHCbz}$) show a 5 to 10-fold difference in rate of alkaline ring opening and different rates of racemization at the α -chiral center. Computer graphics and energy minimization programs are being used to determine the preferred conformations and energies of these isomers, in the hope of being able to identify the basis for this significant difference in steric strain energy.

ANTIDIABETIC DRUGS: ALDOSE REDUCTASE INHIBITORS

Inhibition of the enzyme aldose reductase represents a new pharmacological approach toward the treatment of late-onset diabetic complications. These complications affect the eye, kidney, nervous system and circulation; they are thought to result from hyperosmotic effects of high concentrations of sorbitol, in turn resulting from the reduction of the excess glucose symptomatic of diabetes. Our methods for the synthesis of inhibitors of tryptophan-metabolizing enzymes involve intermediates (XI) which are fairly similar in overall structure to compounds (e.g., Kyorin, XII) now in clinical trials as aldose reductase inhibitors. Furthermore, our kinetic and mechanistic studies have shown that the lactone ring of XI is opened gradually at mildly alkaline pH; should XI bind to aldose reductase, the possibility then exists that the compound might serve as a covalent affinity label for the tyrosine phenolic group present in the inhibitor-binding site and believed to be critical for activity.



The first series of compounds evaluated as inhibitors showed the spiro-lactone (XI) to be active only at concentrations 100 times that of commercial inhibitors; on the other hand, the hydroxyacids (XIII) resulting from ring opening were ca. ten times more active than the lactones, providing a totally new direction for the design of inhibitors. By taking advantage of the lactone-ring strain in XI, it has been possible to synthesize 3-azido equivalents of XIII. We anticipate that such compounds may serve as photoaffinity labels for aldose reductase.

TENUTAUTOMER ANALOGUES

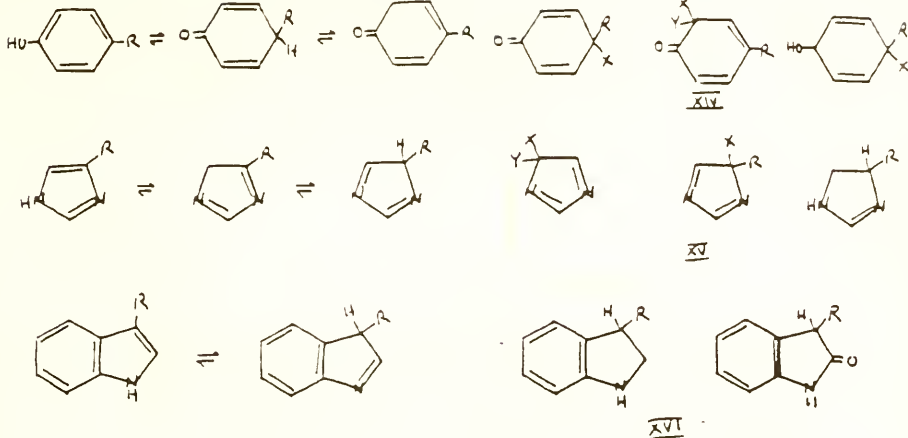
Our studies in tryptophan chemistry and biochemistry have revealed that the molecules present in the active sites of tryptophanase and tryptophan synthase are not the common NH tautomers (tantotautomers) of tryptophan but the higher energy, minor tautomers (tenutautomers). A variety of biological metabolites have similar major and minor capabilities - phenols, catechols, imidazoles, purines, etc. It is conceivable, therefore, that a variety of enzymes utilize an ability to bind and stabilize tenutautomers as a means of activating the substrate for a chemical transformation. We now have ample evidence that tenutautomers are the active species in a number of test-tube reactions of both phenols and imidazoles; furthermore, the experimental data for some enzyme-catalyzed reactions might become more intelligible if the substrates were viewed as their tenutautomers.

Since it is still impossible to examine the detailed structure of a substrate within a binding site, arguments for the tenutautomer concept must be based on evidence and inference from the behavior of stable tenutautomer analogues (XIV, XV). This approach was highly successful and very convincing in the case of tryptophan (XVI). We have undertaken analogous studies for the other tautomeric systems.

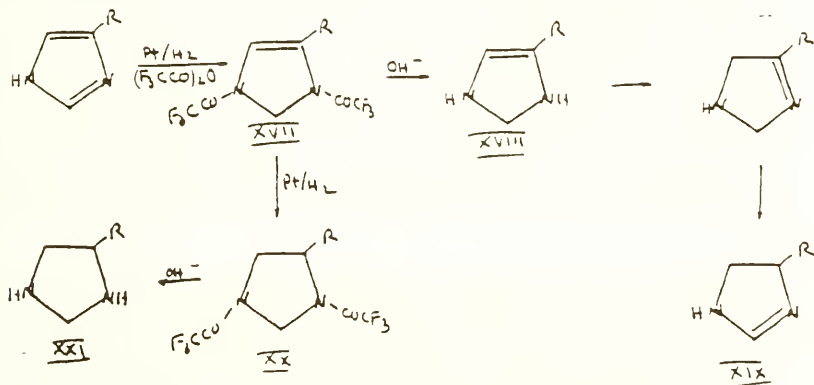
TANTOTAUTOMER

TENUAUTOMERS

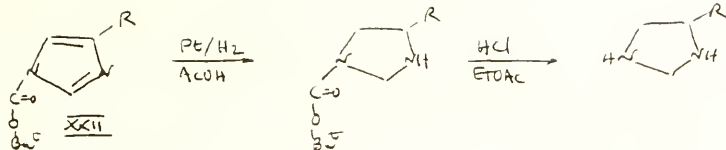
TENUAUTOMER ANALOGUES



The imidazole ring is considerable more refractory to reduction than even benzene. We have found that catalytic hydrogenation can be achieved in the presence of trifluoroacetic anhydride, leading to the acylated dihydro derivative (XVII). The trifluoromethyl groups of XVII can be removed at pH 12-13 to give the free amino acid (XVIII). We then hope to isomerize the double bond of XVIII to form XIX. Continued reduction of XVII gives the fully reduced ring. Both XVIII and the tetrahydrofolate derivative of histidine are expected to serve as inhibitors of tetrahydrofolate reductase.



In order to avoid the problems associated with removal of the trifluoroacetyl groups, as well as to extend this method to histidine reduction in peptides, we have demonstrated that t-butoxycarbonylation and protonation are sufficient to activate the imidazole ring for reduction. Thus, XXII undergoes hydrogenation with platinum in glacial acetic acid to give the tetrahydro derivative. The mildness of this procedure now permits application to peptide hormones.



GENERAL PRINCIPLES OF ENZYME CATALYSIS AND SIMULATION

In order to account for the remarkable catalytic power of enzymes, it is generally considered that the activation free energy (the energy hill which must be surmounted to get from starting material to product) is contributed both by binding of the substrate to the enzyme (step 1) and by chemical manipulation of bound substrate (bond-making and breaking, step 2). Popular opinion holds that most of the activation energy is supplied in step 2: We have proposed, however, that the overall catalytic process can be explained more reasonably if it is assumed that the first step (binding) contributes a more significant, and sometimes major, share of the activation energy. To support this theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen into a single, favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). The compounds undergo intramolecular reactions at rates comparable to those catalyzed by enzymes, sometimes even too fast to measure. Enzymes catalyze many reactions which cannot be observed under mild laboratory conditions. We have shown that our "locked" test-tube analogs can undergo a number of these reactions under physiological conditions of temperature and pH. Thus, one can demonstrate such difficult processes as hydride transfer and displacement of aromatic halogens. Recent work has involved the synthesis of compounds designed (1) to evaluate the flexibility of conformationally frozen carbon chains by ring-ring interconversion and (2) to study steric and electronic effects on ^1H and ^{13}C nmr spectra through space rather than through covalent bonds. Studies with several series of aromatic systems have shown that both reaction kinetics and spectral properties are linearly related to the van der Waals size of crowding substituents. The upper limits of energy-related steric crowding could not be evaluated, however, because of inability to introduce the very bulky iodo substituent. After considerable effort, this goal has now been reached; kinetic and spectral studies are in progress.

As part of our studies of practical applications of stereopopulation control, we are currently exploring the use of o-nitroaryl derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate passage from gut to circulatory system and from circulatory system to brain by temporary masking of charge within the molecule. A number of neutral derivatives of dopamine and L-dopa have been synthesized and are undergoing evaluation as prodrugs for penetration of the blood-brain barrier and as slow-release precursors in the blood stream. Protection from premature oxidation of the catechol function and increased lipophilicity have been introduced by use of the cyclic phenylboronate ester. Model studies show that the boronate is gradually cleaved under physiological conditions. In order to increase the oral effectiveness of polar antibiotics, similar nitroaryl protecting groups are being explored. Several such analogs of penicillin have been synthesized and will be submitted for evaluation.

SECTION ON CARBOHYDRATES

The Section works on the molecular interaction between antigens and monoclonal antibodies. Elucidation of the nature of this interaction is important not only in immunology, but for a general understanding of how receptors and haptens work. Our approach is threefold (see below, A, B, and C.). In addition we are working on development of antibodies against possible epitopes on glycoprotein envelopes of viruses (D).

A. The interaction of ligands, both natural and synthetic, with monoclonal antibodies is evaluated and correlated with epitopes on both the antigen and the protein.

B. Rearranged immunoglobulin genes are cloned with the object of obtaining defined mutations of antibodies by oligonucleotide-directed mutagenesis in order to study the particular contribution to binding by certain amino acid residues in the antibody.

C. The labeling of immunoglobulins is effected by their reaction with specifically designed derivatives of ligands.

D. Synthetic carbohydrate determinants which are part of glycoproteins are derivatized and linked to protein carriers. Antibodies obtained to these immunogens are then obtained, purified and evaluated for their possible binding to known glycoproteins and viruses.

RECENT WORK

Sub A. There is an antipolysaccharide monoclonal immunoglobulin capable of binding to the terminus of its dextran antigen only (see *J. Exp. Med.* 142, 435, 1975; *Carbohydr. Res.* 72, 315, 1979). This type of binding has been referred to as cavity binding. We have evaluated hydrogen bond contributions in the binding, and mapped the subsites of this antibody. For this we prepared a number of complex deoxyfluoro (oligo)- α -D-glucosides (see the report with Dr. P. Kovac as PI), and studied the interaction of these compounds with the antibody.

Sub B. Our Section has studied the interaction of monoclonal anti-galactans and their antigens in a detail unsurpassed for other systems. In order to hone this understanding even more, we have embarked on the development of site-specifically mutated anti-galactan antibodies. Thus we are cloning the heavy (H)-chain gene, and the light (L)-chain gene of IgA X24.

Sub C. We have prepared galacto-oligosaccharides having a reactive diazirino-group in their aglycon moiety. The disaccharide has been covalently linked to IgA X24 by photochemical activation, and studies for its conversion to a ^3H -labeled alcohol which remains attached to the protein, are in progress. In the process of developing the chemistry for this, a new blocking group was reported by us (tert-butyl-diphenylsilylether) which can be selectively removed. In addition a new glycosylation reaction was discovered whereby O-trimethylsilyl glycosides can be facily coupled with O-tert-butyl-diphenylsilyl protected aglycons.

Sub D. An epitope on HIV gp120, suspected to partake in the binding to the T cell receptor, was synthetically prepared. This di-mannopyranosyl ligand was attached to KLH-carrier and used to immunize rabbits. The antibody pool was purified to give the IgG fraction, and this was purified by affinity chromatography to give the anti-di-mannosyl fraction only. This is being evaluated as to its capacity to prevent AIDS virus uptake by CD4⁺ T cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19001-16 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Reactions and Immunochemistry of Carbohydrates

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

PI: Cornelis P.J. Glaudemans, Chief, Section on Carbohydrates NIDDK-LC

Others:	A. E. Rao	Visiting Associate	NIDDK-LC
	E. Nashed	Visiting Associate	NIDDK-LC
	P. Kovac	Visiting Scientist	NIDDK-LC
	G. Perdomo	Visiting Associate	NIDDK-LC
	T. Lin	Visiting Fellow	NIDDK-LC

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Carbohydrates

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5

PROFESSIONAL:

5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The Section works on the molecular interaction between antigens and monoclonal antibodies. Elucidation of the nature of this interaction is important not only in immunology, but for a general understanding of how receptors and haptens work. Our approach is threefold (see below, A, B, and C.). In addition we are working on development of antibodies against possible epitopes on glycoprotein envelopes of viruses (D).

A. The interaction of ligands, both natural and synthetic, with monoclonal antibodies is evaluated and correlated with epitopes on both the antigen and the protein.

B. Rearranged immunoglobulin genes are cloned with the object of obtaining defined mutations of antibodies by oligonucleotide-directed mutagenesis in order to study the particular contribution to binding by certain amino acid residues in the antibody.

C. The labeling of immunoglobulins is effected by their reaction with specifically designed derivatives of ligands.

D. Synthetic carbohydrate determinants which are part of glycoproteins are derivatized and linked to protein carriers. Antibodies obtained to these immunogens are then obtained, purified and evaluated for their possible binding to known glycoproteins and viruses.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19003-01 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Synthesis of immunodeterminants

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

PI:	Pavol Kovac	Visiting Scientist	NIDDK-LC
Others:	C.P.J. Glaudemans	Chief, Section on Carbohydrates	NIDDK-LC
	G. Perdomo	Visiting Associate	NIDDK-LC
	T. Lin	Visiting Fellow	NIDDK-LC

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

.5

PROFESSIONAL:

.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The determination of the size of the binding site of an immunoglobulin and the investigation of the nature of forces involved in the binding process requires a large number of ligands. In the case of carbohydrate specific antibodies, ligands constitute series of homologous carbohydrate derivatives which are invariably obtained by sophisticated chemical syntheses. In addition to mono and oligosaccharides related to the natural antigen, we synthesize modified ligands. To those belong molecules having, for example, certain hydroxyl groups replaced by functions which modify the binding pattern, thus allowing to draw conclusions about the importance of various forces in the binding process. To investigate the possible role of hydrogen bonding in the interaction of carbohydrate antigens and antibodies our endeavors are concentrated at synthesizing ligands bearing deoxy and deoxyfluoro functions. Among these, of special interest are derivatives of methyl- α -D-glucopyranoside bearing bulky substituents at position 2 or 3. From the mode of interaction of this type of ligands we expected to be able to draw conclusions about the spatial accessibility of the highest-binding subsite.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19401-23 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Natural Products as Agonists, Antagonists, Desensitizers & Probes for Receptors

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

PI: Dr. Bernhard Witkop

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Metabolites

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

INACTIVE

NOTICE OF INTRAMURAL RESEARCH PROJECT

701 DK 19603-12 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Histidine Analogs

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

PI: Louis A. Cohen Chief, Section on Biochem. Mech. NIDDK-LC

Others: V. Labroo Visiting Associate (Term. 4/23/88) NIDDK-LC

N. Kolodny Visiting Fellow NIDDK-LC

S. Von Hof Visiting Fellow NIDDK-LC

B. Avramovitch Visiting Fellow

COOPERATING UNITS (if any)

G. Feuerstein, Dept. of Pharmacology, USHUH; E. De Clercq, Louvain, Belgium;
H. Kimoto, Nagoya, Japan; R. Howard, DNAX, Palo Alto, CA

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.1

PROFESSIONAL:

1.4

OTHER:

0.7

CHECK APPROPRIATE BOX(ES)

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

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

TRH Analogs: In addition to governing the release of thyrotropin and prolactin in the pituitary gland, TRH (L-pyroglutamyl-L-histidyl-L-proline amide) is known to possess a wide variety of effects on both the central nervous system (CNS) and the cardiovascular system (CVS). TRH has shown promise for use in the treatment of shock, as an analeptic and antidepressant, and as a promoter of the regeneration of injured spinal cord. However, the great variety of its biological effects presents a serious drawback to its use as a specific drug. Our early studies with synthetic analogs of TRH (involving modification of the imidazole ring of histidine) has suggested that the peptide hormone elicits each of its physiological responses at a different receptor and that appropriate analogs may achieve some of the desired specificity of action.

It is now quite clear that at least four of the biological activities of TRH involve uniquely different receptors and that, after a decade of effort in various laboratories, the separation of these activities has at last been achieved. Thus, 4-NO₂-Im-TRH, high selective for CVS activity, may be useful in the treatment of various forms of shock without a concomitant enhancement of thyroid activity or of prolactin release. On the other hand 2,4-I₂-Im-TRH or Nva⁴-TRH may be useful as diagnostic tools for the assessment of pituitary function without the risk of increased blood pressure and tachycardia induced by TRH. The iodinated analog is particularly useful since it can be prepared readily with radioactive iodine. Furthermore, each of these selective agonists should provide a useful research tool for the study of the role and mechanism of TRH involvement in the respective function.

Binding studies in rat brain tissue show that these analogs bind only weakly or not at all. Thus, we must search for non-TRH receptors or nonreceptor mechanisms to explain the potent cardiovascular activity of some of these analogs.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19604-18 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

General Principles of Enzyme Catalysis and Simulation

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

PI: Louis A. Cohen Chief, Sec. Biochem. Mechanisms NIDDK-LC

Other: Michael King Guest Worker NIDDK-LC

COOPERATING UNITS (if any)

Yoshio Ueno, Nagoya, Japan; Wieslaw Antkowiak, Poznan, Poland;
Yoshio Takeuchi, Toyama, Japan

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.5

PROFESSIONAL:

0.3

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

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

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

In order to account for the remarkable catalytic power of enzymes, it is generally considered that the activation free energy is contributed both by binding of the substrate to the enzyme (step 1) and by chemical manipulation of the bound substrate (bond-making and breaking, step 2). Popular opinion holds that most of the activation energy is supplied in step 2: We have proposed, however, that the overall catalytic process can be explained more reasonable if it is assumed that the first step (binding) contributes a more significant, and sometimes major, share of the activation energy. To support this theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen into a single, favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). These compounds undergo intramolecular reactions at rates comparable to those catalyzed by enzymes, and show that the protein raises both the entropic and enthalpic components of the substrate by binding it in a single, rigid conformation. Recent work has involved the synthesis of compounds designed (1) to evaluate the flexibility of conformationally frozen carbon chains by ring-ring interconversion and (2) to study steric and electronic effects of ^1H and ^{13}C nmr spectra through space rather than through covalent bonds. Studies with several series of aromatic systems have shown that both reaction kinetics and spectral properties are linearly related to the van der Waals size of crowding substituents. The upper limits of energy-related steric crowding could not be evaluated, however, because of inability to introduce the very bulky iodo substituent. After considerable effort, this goal has now been reached; kinetic and spectral studies are in progress. As part of our studies of practical application of stereopopulation control, we are currently exploring the use of o-nitroaryl derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate passage from gut to circulatory system to brain by temporary masking of charge within the molecule.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 19605-12 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Chemistry of Substituted Imidazoles

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

PI: Louis A. Cohen Chief, Section on Biochem. Mechanisms NIDDK-LC

Others: B. Avramovitch Visiting Fellow NIDDK-LC

N. Kolodny Visiting Fellow NIDDK-LC

COOPERATING UNITS (if any)

H. Kimoto, Industrial Res. Inst., Nagoya, Japan; R. Henkin, Georgetown Univ. Hosp., Wash., D. C.; E. DeClercq, Louvain Univ., Belgium; A. Shanzer, Rehovot, Israel; W. Nagai, Nagoya, Japan

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.2

PROFESSIONAL:

1.5

OTHER:

0.7

CHECK APPROPRIATE BOX(ES)

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

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

We have now developed procedures for the conversion of aminohistidines into azido and nitrohistidine, of amino to chloro, bromo and iodo, of trifluoromethyl into methyl, cyano, carboxy, carbomethoxy, etc. Recently, we synthesized 2- and 4-(pentafluoroethyl)-histidines by photochemical radical substitution. The compounds are converted by base into the corresponding (trifluoroacetyl)histidines, which have such reactive carbonyl groups that they may serve as affinity labels for histidine-binding sites. The trifluoroacetylimidazoles can be reduced to the secondary alcohols, also obtainable by direct condensation of imidazoles with trifluoroacetaldehyde. In turn, the secondary alcohols can be oxidized to the trifluoroacetyl ketones. Upon treatment with methanolic base, (trifluoromethyl)histidine can be converted into (trimethoxymethyl)histidine and pentafluoroethyl into the corresponding ketal. These ortho functionalities are also of interest as potential covalent affinity labels.

Ring-trifluoromethylated imidazoles show the unique property of losing hydrogen fluoride above pH 8 to form metastable difluorodiazafulvenes, which then react with any available nucleophile to form new covalent bonds. Such intermediates, derived from trifluoromethylhistamine or histidine, may be able to serve as covalent affinity labels for specific binding sites, both *in vitro* and *in vivo*. It would be desirable, therefore, to have available a series of trifluoromethyl analogs with a range of reactivities, and to be able to correlate reactivity with some substituent parameter. Our discovery of a simple photochemical method for the trifluoromethylation of imidazoles has made available a large series of analogs for study.

The long-sought 2-bromo and 2-iodoimidazoles can now be obtained by simple reduction of the 2,4-dihalo compounds with hot hydrochloric acid. In the case of the dihalohistamine or histidine, hot water is sufficient to achieve selective reduction.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 19606-12 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Halogenated Biogenic Amines in Biochemistry and Pharmacology

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

PI: Kenneth L. Kirk	Research Chemist	NIDDK-LC
Other: A. Adejare	Visiting Fellow	NIDDK-LC
D. Furlano	IRTA Fellow	NIDDK-LC
C. Diane Truie	IRTA Fellow	NIDDK-LC
K. Jacobson	Sr. Staff Fellow	NIDDK-LC

COOPERATING UNITS (if any)

JDaly; CRCreveling; FGusovsky (LBC, NIDDK); MChanning; DKiesewetter; R Finn (CC, Dept. Nucl. Medicine); DSGoldstein; GEisenhofer (HE, NHLBI); KJKopin (DIR, NINCDS); MLinnoila (NUAAA); CChieuh (NIMH); SReppert (Harv. Med. Sch.); GRudnik (Yale Univ.); KAMuszkat (Weizmann Inst.); VWPike (MRC Cyclotron Unit, Hammersmith Hosp. London)

LAB BRANCH

Laboratory of Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland, 20892

TOTAL MAN-YEARS

4.8

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Biogenic amines play key roles in neurotransmission, metabolism, and in control of various physiological processes. Ring-fluorinated analogs have proved to be powerful tools for the study of the mechanisms of transport, storage, release, metabolism, and modes of action of these amines since they simulate the geometries of the natural compounds so well. By virtue of its very small size and high electronegativity, fluorine is a very favorable replacement for hydrogen in these analogs. In 1970, we developed novel methods for the introduction of fluorine into organic molecules and have applied these methods to the syntheses of a wide variety of biogenic amines with fluorine at various ring-positions. The biological properties and usefulness of these ring-fluorinated biogenic amines have proved to be extremely rewarding and continue to find applications in a multitude of studies. Of particular significance was the discovery that 6-fluoronorepinephrine is a selective alpha-adrenergic agonist and 2-fluoronorepinephrine is a selective beta-adrenergic agonist. Various explanations for the role of fluorine in creating such selectivities have been considered and discarded. Proposals under current consideration include a critical dipole-dipole repulsion between the benzylic hydroxyl group and fluorine in the 2- and 6-positions. This interaction could lead to side-chain conformation preferences favorable for interaction with the beta and alpha adrenergic receptors, respectively. Effects of fluorine on the electronic properties of the aromatic ring are considered also to be important in defining selectivities. Experiments to differentiate between conformational and electronic effects have been initiated. Biological properties of new analogs, including fluorinated epinephrines have mechanistic significance. The use of ^{18}F -labeled 6-fluorodopamine, the biological precursor to 6-fluoronorepinephrine, has been found to be an excellent scanning agent for peripheral noradrenergic innervation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19607-06 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Chemistry, Biochemistry and Pharmacology of Bioindole Analogs

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

PI: Louis A. Cohen Chief, Section on Biochem. Mech. NIDDK-LC

Other: Rita Labroo Guest Worker GWU

COOPERATING UNITS (if any)

Edith Miles, LBP, NIDDK; Robert Phillips, University of Georgia; Peter Kador, LMOD, NEI; Hiroshi Kimoto, Nagoya, Japan

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.2

PROFESSIONAL:

1.0

OTHER:

.2

CHECK APPROPRIATE BOX(ES)

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

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

Studies on the mechanisms of certain tryptophan reactions suggest that the indolenine tautomer should be the true substrate for some tryptophan-metabolizing enzymes. The first conclusive support for this concept is found in our demonstration that 2,3-dihydro-L-tryptophan and oxindolyl-L-alanine, analogs of the indolenine tautomer of tryptophan (tetrahedral carbon at C-3), are potent competitive inhibitors of tryptophan synthase and tryptophanase. Furthermore, the two enzymes show opposing specificity for the C-3 diastereoisomers of 2,3-dihydro-L-tryptophan, suggesting that these enzymes catalyze their reactions via enantiomeric indolenine intermediates. Dioxindolyl-L-alanine (3R) is also an inhibitor of tryptophanase and matches the stereochemistry of (3R)-2,3-dihydro-L-tryptophan, which inhibits the same enzyme. 3-Azido-oxindolyl-L-alanine, a potential photo-affinity label for tryptophanase, has been prepared.

Inhibition of the enzyme aldose reductase represents a new pharmacological approach toward the treatment of late-onset diabetic complications. These complications affect the eye, kidney, nervous system and circulation; they are thought to result from the hyperosmotic effects of high concentrations of sorbitol, in turn resulting from the reduction of the excess glucose symptomatic of diabetes. Our methods for the synthesis of inhibitors of tryptophan-metabolizing enzymes involve spiro lactone intermediates which are fairly similar in overall structure to compounds now in clinical trials as aldose reductase inhibitors.

The first series of compounds tested show the spiro lactones to be active only at concentrations 100 times those of commercial inhibitors; however, the hydroxyacids are ten times more active than the lactones. Replacement of the hydroxyl group by halogen and azido provides compounds which may act as affinity and photo-affinity labels.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19608-05 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Functionalized Congeners of Bioactive Compounds

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

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Other: B. Bradbury IRTA Fellow NIDDK-LC

K. Kirk Research Chemist NIDDK-LC

J. Zimmet Student Volunteer NIDDK-LC

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.7

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

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

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

Recent work in our laboratory and in others has demonstrated that certain drugs may be attached to well-defined "carrier" molecules and still retain the ability to bind to the receptor site and effect biological activity. This synthetic strategy for the attachment of drugs to carriers is termed the "functionalized congener" approach. The "carrier" molecule may be many times larger than the parent drug; indeed there is practically no maximum size limitation for a fully potent analog. Unlike the prodrug approach or the immobilization of drugs for slow release, the "functionalized congener" approach is designed to produce analogs for which no metabolic cleavage step is necessary for activation. Moreover, the attachment of the drug to a "carrier" such as a peptide may result in the enhanced affinity at an extra-cellular receptor site and an improvement in the pharmacological profile of the parent drug.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19609-04 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Determination of Amines and Amine Metabolites in Biological Samples

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

PI: Kenneth L. Kirk Research Chemist NIDDK-LC

Other: K. A. Jacobson Sr. Staff Fellow NIDDK-LC

COOPERATING UNITS (if any)

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G. Gusovsky, NIDDK, NIH; A. Gjerris (Copenhagen)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been temporarily discontinued.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19610-01 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Prosthetic Groups for Radiolabeling of Functionalized Drugs and Peptides

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

PI:	K. Jacobson	Senior Staff Fellow	NIDDK-LC
Other:	K. Kirk	Research Chemist	NIDDK-LC
	Y. Shai	Visiting Associate	NIDDK-LC
	S. Barone	Special Volunteer	NIDDK-LC

COOPERATING UNITS (if any)

J. Roth (NIDDK), M. Lesniak (NIDDK), R. Finn (NM-CC),
M. Channing (NM-CC), D. Kiesewetter (NM-CC), J. Daly (NIDDK)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.6

PROFESSIONAL:

1.6

OTHER:

CHECK APPROPRIATE BOXES)

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

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

The use of radioisotopes to label organic compounds for use in diagnostic nuclear medicine is well documented in the literature. It has been found that certain radiolabeled compounds will localize in the brain, heart, or in other target organs or tissues to a sufficient level to allow for imaging thereof. There has been increasing interest in finding compounds which will more effectively cross the blood-brain barrier, thus facilitating more efficacious imaging of the brain.

Binding sites for certain drugs in an animal or organ may be localized as a result of the synthesis of high specific activity radiolabeled analogs which have high affinity for that binding site. Prosthetic groups may be attached to a drug or other receptor ligand for the purpose of efficient and selective chemical capture of a particular radioisotope. Having developed functionalized congeners of theophylline and other drugs acting at adenosine receptors, we are now developing prosthetic groups for radioisotopes such as ^{18}F , ^{123}I , and ^{125}I , to be coupled to these functionalized drug molecules. The prosthetic groups contain amino or carboxylic groups which are to be condensed covalently to functionalized drugs to give conjugates of high affinity at a particular receptor.

Positron emission tomography (PET) has been used for imaging receptors in the brain and other organs. A prosthetic group for chemical capture of ^{18}F requires rapid and efficient reaction and purification, since the half-life is only 110 minutes.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19611-01 LC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Development of Drugs Acting at Adenosine Receptors

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

PI:	K. Jacobson	Senior Staff Fellow	NIDDK-LC
Other:	K. Kirk	Research Chemist	NIDDK-LC
	J. Zimmet	Student Volunteer	NIDDK-LC
	S. Barone	Special Volunteer	NIDDK-LC
	U. Kammula	Special Volunteer	NIDDK-LC

COOPERATING UNITS (if any) J. Daly (NIDDK), T. Seale (Univ. Okla.), B. Fredholm (Karolinska Inst.), J. Neumeyer (Res. Biochem., Inc.), P. Churchill (Wayne State), G. Stiles (Duke Univ.), P. Marangos (NIH), M. Williams (CIBA-GEIGY), G. Evoniuk (NIDDK-LN)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.6

PROFESSIONAL:

1.1

OTHER:

.5

CHECK APPROPRIATE BOX(ES)

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

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

The extracellular adenosine receptor has a modulatory role in the nervous, circulatory, endocrine, and immunological systems. The prospect of harnessing these effects specifically for therapeutic purposes is attractive.

A functionalized congener approach to drug design has been applied to the adenosine receptor to produce analogs of agonists and antagonists which have promise as therapeutic agents and as receptor probes. In the antagonist series new analogs which combine potency, water solubility, and A₁-adenosine receptor selectivity in the same compound are now being evaluated in in vivo testing.

The Laboratory of Cell Biology and Genetics carries on a broad program of investigation into hormone and transmitter secretion and the molecular events regulating these processes. Four specific tissues are used: chromaffin cells, which secrete adrenaline, ATP and endogenous opiates; pancreatic beta cells, which secrete insulin; the frog neuromuscular junction, in which acetylcholine is the principal secreted substance, and bovine submucosal glands from the trachea, which secrete mucins.

Membrane fusion is a key event in numerous biological processes, including neurotransmission and exocytosis, and may depend on calcium and other ions and factors. Much of our work this year has been devoted to biological signals which regulate this process in chromaffin and B cells, as well as to studies on the calcium binding protein synexin. At present we consider synexin, to be at least one likely mediator of membrane contact and fusion during exocytosis.

This year we have learned much more about the molecular basis of how synexin might go about fusing membranes to one another. Based on preliminary genetic and physical data we hypothesized that synexin might enter into fusing membrane partners simultaneously, thereby providing a hydrophobic bridge across which phospholipids might cross and mix. As partial tests of this hypothesis we examined whether synexin might actually insert into the substance of the bilayer, using a capacitance technique. We found unequivocal evidence that synexin indeed entered the bilayer. In later studies we determined that synexin placed into acidic phospholipid bilayers at the tip of a patch pipet could also act as voltage-sensitive calcium channels. We interpreted this to mean that synexin not only entered the membrane but also spanned the membrane. Synexin channels conducted primarily calcium, being only 1-2% as selective for barium.

The exocytosis process, of course, is much more than the ultimate step of membrane fusion, and we have examined the process of secretion from chromaffin cells from a variety of points of view. One useful technique, developed here in our laboratory, has been to follow secretion on-line using ATP secreted from the granules into an extracellular medium containing luciferin-luciferase. We found that when chromaffin cells were stimulated with acetylcholine the characteristic delay between stimulation and the onset of secretion was reduced as the temperature was increased, or if the concentration of acetylcholine were increased. This means that a variety of kinetically distinguishable events must occur between binding of the secretagogue and exocytosis. The possible involvement in cyclic nucleotides, and GTP analogues has been studied by adding relatively permeable cGMP and other G nucleotides to cells prior to stimulation. We found that pre treatment of cells with 8-Bromo cyclic GMP potentiated ATP secretion induced by acetylcholine, potassium and barium.

The possibility that various G-nucleotides might regulate secretion lead us to investigate the possible contribution of intracellular calcium stores to this process. We had previously noted that muscarinic receptors potentiated nicotinic receptor-activated secretion. We found recently that caffeine, a known releaser of calcium from the sarcoplasmic reticulum of muscle and other cell types, also caused mobilization of calcium

from internal stores using the dye FURA-2 as a detector. We also found that caffeine modestly potentiated secretion from the chromaffin cells.

Now knowing that extracellular calcium was the primary source of calcium for control of secretion, we turned our attention to mechanisms by which calcium entered the cell. Calcium enters chromaffin cells through the acetylcholine receptor-gated channel, but it also enters through voltage-sensitive channels. We determined that depolarization opened dihydropyridine-sensitive channels, but that perhaps 25-30% of calcium entered the cell through drug-insensitive channels. We also noted that secretion was not entirely blocked by these drugs, and concluded that chromaffin cells must contain in addition to the standard voltage sensitive L-type channel, a version of the L-type channel that was insensitive, not only to dihydropyridine drugs, but also *w-conotoxin*.

The importance of the cytosolic calcium ion concentration was also made manifest by our recent study on the mechanism of action of certain peptide inhibitors of metalloendopeptidases. These inhibitors had been noted to interfere with secretion and membrane fusion from a variety of cell types, and naturally it had been assumed that the appropriate proteolytic enzymes might be responsible for the fusion or secretion processes. However, a detailed study revealed rather that these inhibitors suppressed secretion by accelerating the efflux of calcium from the cell.

The intracellular pH has been implicated in regulation of secretion in a variety of cells, and we also examined chromaffin cells from this viewpoint. We found that administration of ammonia to chromaffin cells lowered the cytosolic pH, but that this change had little or no consequence on cytosolic free calcium ion concentration. Nonetheless, ammonia inhibited secretion by ca. 50%. The basis of this inhibition appears to be the alkalinization of the chromaffin granule interior, since maresin, which alkalinizes the granule interior but acidifies the cytosol, also inhibited secretion by the same amount.

While calcium and protons can be measured with some accuracy in the volume of the whole cell, the detailed distribution of these and other elements in different parts of the cell can only be determined by electron probe microanalysis. We performed such an analysis on thin frozen sections of chromaffin cells, and learned that the nucleus, cytoplasm, mitochondria and granules had different water contents. However, knowing these values exactly we were able to measure the true concentrations of ions in the different compartments, and to determine changes over millisecond time intervals during isolation of granules, stimulation of cells and effects of a variety of drugs.

Some of these conclusions have been examined in another secreting cell, the insulin-secreting B-cells of the Islets of Langerhans. In this cell type secretion depends on calcium, and depolarization of the cell by glucose is accomplished by closing potassium channels. The mechanism is not known, and has been the subject of enormous efforts around the world. B-cells also contain an ATP-blockable K channel, which has been implicated in regulation of insulin secretion. For example, tolbutamide blocks this channel and evokes secretion in normal islets, as well as in islets from patients with the initial stages of type II diabetes. We learned that fetal B cells from rats possessed functional ATP-blockable channels,

but still secreted insulin poorly in response to glucose. Therefore, the ATP-blockable K channel is not primarily for glucose-coupling to secretion via metabolites.

Other aspects of regulation of insulin release, as in the chromaffin cell, involve muscarinic receptor action. Acetylcholine potentiates glucose-induced insulin release, and the mechanism appears to be via M1 type muscarinic receptors. The potentiation process appears to be by acetylcholine-evoked depolarization, thus implicating a specific potassium channel in the process.

In addition to acetylcholine, peptide hormones also appear to exert modulating action on insulin secretion. The insulinotropic gastric inhibitory peptide (GIP), for example, suppresses insulin secretion at low glucose concentrations but enhances insulin secretion at high glucose concentrations. Thus, GIP limits possible hypoglycemia during hypoglycemia, but acts in the opposite manner during hyperglycemia to enhance insulin secretion and bring glucose concentration back to "normal" levels. The possible pathologic state of this regulatory system in human diabetic states remains to be thoroughly explored.

The ability of B-cells in the islets of Langerhans appears to depend on the fact that the B-cells are coupled to each other, and possibly to other cell types. Indeed, single B-cells appear to be poor secretors of insulin. We found that the physiologic activator of insulin secretion, CAMP, may work by direct action on the coupling mechanism. For example, we found that forskolin, an activator of adenylate cyclase, enhanced electrical coupling between neighboring B-cells in islets.

All endocrine cells in the body secrete their products through or around endothelial cells of proximal capillaries. We learned that chromaffin granule products are able to cause endothelial cells cultured from the adrenal medulla to synthesize and secrete prostacyclin. The activity was mainly due to ATP, acting through a P₂ purinergic receptor. Since prostacyclin causes blood vessels to dilate, we have concluded that the action of secreted ATP must include enhancing the efficiency of the pathway for hormones out of the gland and into the circulation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 21008-22 LCBG

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Cytogenetics

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

P.I.: J.H. Tjio Chief, Section on Cytogenetics, LCBG, NIDDK

COOPERATING UNITS (if any) Albany Med. College, Albany, NY (E.S. Raveche), Univ. of California, Berkeley (G. Brecher), Ernst Moritz Arendt Universitat, Greifswald, DDR (F. Herrmann), Karl Marc Universitat, Leipzig, DDR (H. Storch), Humboldt Univ. Zoologisches Museum, East Berlin (S. Santibanez), Univ. Buenos Aires (D. Gomez)

LAB/BRANCH

Laboratory of Cell Biology and Genetics

SECTION

Cytogenetics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1

PROFESSIONAL:

1

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

Summary of Work:

1. Sex reversal studies in fresh water fishes.
2. In vivo effects of hyperdiploid Lyl⁺ B cells.
3. Influence of host environment on growth of clonal Lyl⁺ B cell.
4. Serial passage of bone marrow stem cells through murine hosts.
5. Observations of chromosomal aberrations in lymphoid tissue cultures.
6. Adhesion invasion and metastases in mouse.
7. Post and prenatal diagnosis of chromosomal aberration of cultured amniotic fluid cells and CVS.
8. Cytotaxonomic studies of the family Anabantidae.

NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 DK 21019-06 LCBG

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Mechanism of hormone and transmitter secretion

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

P.I.: Harvey B. Pollard, Chief, Laboratory of Cell Biology and Genetics, NIDDK
 Others: R. Ornberg, Ph.D., Elec. Microscopist; G. Lee, Ph.D., Res. Chemist; E. Rojas, Ph.D., VS; I. Atwater, Ph.D., Expert; R. Santos, VA; M. Levine, M.D., RA; K. Brocklehurst, Ph.D., VF; P. Lelkes, Ph.D., VS; L. Rosario, Ph.D., VA; E. Forsberg, Ph.D., SF; A. Burns, Ph.D., Expert; I. Cabantchik, Ph.D., SV; A. Stutzin, Ph.D., VF; J. Bitran, MD, VF; M. Srivastava, Ph.D., VF; C. McCutchen, Ph.D., Res. Phys; G. Kuijpers, Ph.D., VF; K. Magendzo-Weinberger, Ph.D., SV; P. Mathias, M.D., VF; A. Munoz, M.D., SV; V. Vena, Ph.D., M.D., VA; M. Li, M.D., VF; Y. Shi, Ph.D., SV; G. Goping, EM Tech.; B. Cheung, SV; Carroll, P., NRSA, SV; Bdoлах, A., Ph.D., SV; P. Vanek, SV; R. Pelz, SV; D. Tombaccini, M.D., SV; W. Hartzell, SV; P. Washko, P. SV

COOPERATING UNITS (if any)

Dipak Banerjee, Ph.D., University of Puerto Rico; G.D. Pappas, Ph.D., Univ. of Illinois

LAB/BRANCH

Laboratory of Cell Biology and Genetics

SECTION

Cell Biology and Biochemistry

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS

33

PROFESSIONAL

33

OTHER

0

CHECK APPROPRIATE BOX(ES)

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

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

Our recent work has focussed on the processes leading to fusion between granule and plasma membranes during exocytosis in cells such as chromaffin cells, beta cells in islets of Langerhans, frog neuromuscular junction, and tracheal submucosal gland cells. Synexin, a calcium binding membrane fusion protein found in many tissues, was found to fuse membranes by a mechanism involving membrane mixing occurring prior to volume mixing. Synexin was also found to enter membranes and change the capacitance, and simultaneously to exhibit calcium channel activity. From these and other data we have formulated a hydrophobic bridge hypothesis for membrane fusion driven by synexin. The fast-freeze electron microscopy technique has been used to measure changes in distribution of elements and their concentrations in discrete regions of cytosol alkalization of chromaffin granules suppresses secretion, implicating the state of the granule interior in the exocytosis process. Metalloendoprotease inhibitors block secretions by accelerating calcium efflux. Calcium enters chromaffin cells through a variety of voltage-sensitive calcium channels, including at least one that is insensitive to dihydropyridines or w-conotoxin. In islet of Langerhans, fetal B-cells have functional ATP blockable channels, but still respond poorly to glucose. Cyclic Amp may potentiate insulin secretion by enhancing coupling between cells. Potassium channels from internal organelles of nerve endings can be reconstituted using a patch pipet technique.

I. POLYAMINES

Polyamines are major cellular components, and have been shown to be involved in many systems related to growth and differentiation. Our studies have been directed towards elucidating the physiological function, biosynthesis, and regulation of these amines. During the past year our studies have been directed toward a study of S-adenosylmethionine decarboxylase, which is a key enzyme in the biosynthesis of spermidine and of spermine in both prokaryote and eukaryote cells. This enzyme has a most unusual structure, as it contains a covalently linked pyruvoyl moiety which is required for enzyme activity. This enzyme is coded for by the speD gene.

Escherichia coli. We have continued our studies on the nucleotide sequence of the speEspeD operon, and have defined the operator and terminator regions, as well as the structural genes. We have defined the position of this operon on the physical map as 138-140 Kbp clockwise to the original of the E. coli chromosome. We have shown that the enzyme S-adenosylmethionine decarboxylase is first formed as a proenzyme that is processed by a post-translational cleavage reaction at a Lys111Ser112 peptide bond. By site-specific mutagenesis we have prepared mutations in the Lys111 position, and have shown that the processing is markedly decreased or eliminated by amino acid substitutions for the Lys111 amino acid. We have also shown that the processing can be demonstrated in cell-free extracts. Conditions have been developed for the overproduction of the enzyme in plasmid-containing strains. S-Adenosylmethionine decarboxylase represents 10% of the cellular protein in such strains, and can be easily purified to homogeneity.

Saccharomyces cerevisiae. We have cloned and sequenced the gene for S-adenosylmethionine decarboxylase (speE) in yeast. The gene has been placed in a multicopy shuttle vector, and strains carrying this vector have been shown to produce 50 times more enzyme than a wild type strain.

. Drs. C. W. Tabor, H. Tabor, Q.-W. Xie, and K. Kashiwagi

II. YEAST RNA VIROLOGY

There are five families of double-stranded RNA (dsRNA) virus-like particles (L-A, L-BC, M, T, and W) and two distinct single-stranded RNA (ssRNA) virus-like entities (20S RNA and 23S RNA) that replicate in cells of Saccharomyces cerevisiae. We have studied how these genomes replicate in yeast with emphasis on the role of the host. We have shown that over 30 chromosomal genes (MAK genes) are necessary for dsRNA replication, while over 6 chromosomal genes (SKI genes) repress this replication. Highly purified virus-like particles (VLPs) carry out both (+) strand and (-) strand synthesis of L-A, L-BC, or M RNA in vitro in a conservative, sequential reaction.

We developed an in vitro replication system [(-) strand synthesis on a viral (+) strand template producing dsRNA] for the L-A system. Using isolated opened empty viral particles, we demonstrated site-specific binding of viral (+) strands. The internal sequence 5' UUUGGCCAGG 3' determines this binding. In addition to this sequence, the replicase reaction requires the correct 3' end, but not the 5' end. The viral 180 kDa minor protein binds ssRNA and is encoded by L-A, having an N-terminal major coat protein domain and a C-terminal region responsible for ssRNA binding. We have cloned and sequenced the L-A genome and find that the 180 kDa protein is encoded by the fusion of two overlapping ORFs, ORF1 and ORF2. ORF1 encodes the major coat protein, and ORF2 encodes the ssRNA binding domain of the 180 kDa protein. ORF2 has substantial homology with viral RNA polymerases of picornaviruses and togaviruses. We have designed an RNA virus-based vector for yeast using our clone of X dsRNA, a deletion derivative of L-A dsRNA.

We have cloned and sequenced the MAK16 and SKI3 genes. Both are localized in the nucleus. The SKI3 gene encodes a 160 kDa protein whose only essential function for the cell is repression of viral replication. The MAK16 protein is involved in both viral replication and is essential for progression of the cell cycle with mutants arresting in the G₁ phase.

. . . . Drs. R. B. Wickner, T. Fujimura, R. Esteban, T. Ichio, S. K. Rhee, and Y. Matsumoto

III. NUCLEIC ACIDS

L Transposons

Introduction. All mammals contain several families of repetitive DNA sequences that comprise a substantial portion of the genome. Our studies on one of these families, the rat long interspersed repeated DNA family (LINE or L family) of the rat have provided, among other things, the first direct evidence that a mammalian highly repeated DNA family consists of mobile DNA elements; the presence or absence of L members causes allelic variation at a number of single copy loci. For this reason and because it appears as if L elements of mammals quite likely are the mammalian analogs of the I elements of *Drosophila*, a family of bonafide mobile elements, we think it appropriate to refer to the mammalian L family as L transposons. The L transposon of rats contains about 40,000 members and accounts for about 10% of the rat genome. Most members are full length (6.7 kb), 5 kb of which is devoted to protein encoding sequence. A promoter-like sequence for the transcription of the open reading frames (ORFs) is at the left end of the element, and G-rich homopurine stretches are at the other end. L elements terminate about 35 bp 3' of the G-rich (GHP) stretches in an A-rich region of variable length.

Current Findings. Studies on the structure and effect of a guanine-rich homopurine sequence, which is a conserved feature of mammalian L transposable elements, showed that this type of structure induces unpairing of contiguous duplex DNA that would ordinarily be stably basepaired.

The unpaired duplex can take up oligonucleotide which can be extended by added DNA polymerase. Therefore, guanine-rich homopurine sequences predispose contiguous DNA sequences to the first two steps of genetic recombination. Since guanine-rich homopurine sequences are present in a number of regions in the eukaryotic genome, these findings have implications beyond those pertaining to the biological properties of L DNA elements.

In order to understand how guanine-rich homopurine sequences unpair contiguous DNA, we also examined the structure of this sequence itself, using a variety of chemical and enzymatic probes for DNA structure. Our results showed that this sequence, although about 70% G + C, contains significant single-stranded quality; i.e., it is functionally unpaired. This explains in a simple and straightforward way the effect of the homopurine sequence on contiguous DNA and also several other properties that have been observed for homopurine stretches. Furthermore, our studies ruled out a number of other structures that have been proposed for homopurine sequences.

. . . . Drs. K. Usdin and A. V. Furano

We have determined the DNA structure of the left 1.5 kb of two newly isolated full length members of the rat L DNA family (long interspersed repeated DNA). In contrast to earlier isolated L members, both of these contain a 650 bp promoter region that is most likely full length. In addition, the promoter of both members has undergone a partial tandem duplication. A second internal region of the left end of one of the reported members is also tandemly duplicated. Since other regions of L DNA do not contain tandemly repeated sequences, the left end of the rat L element is particularly prone to this type of genetic rearrangement. Interestingly, the promoter region of the mouse L element, which is completely distinct in both DNA sequence and length from the rat L DNA promoter, is also subject to tandem duplication. It is possible that the susceptibility to genetic rearrangement of the left end of mammalian elements is related to the fact that during evolution the otherwise conserved mammalian L DNA families have each acquired completely different promoter-like regions.

. . . . Drs. A. V. Furano, S. M. Robb, and F. T. Robb

We found that the 650 bp promoter-like region at the left end of one of the newly isolated and characterized rat L DNA elements described above can activate the prokaryotic chloramphenicol acyl transferase gene in a rat cell line. Activation only occurs when the promoter region is oriented to the transferase gene as it is to the L DNA protein-encoding sequences and is 75% inhibited by methylation of just 5 of the 22 CpGs present in the promoter. The G + C rich promoter contains enough CpGs to qualify it as a CpG island, but in contrast to other CpG islands, genomic L DNA promoters are fully methylated in both somatic cell and sperm DNA as judged by restriction enzyme analysis. Partial

demethylation of the genomic promoters by treatment with 5-azacytidine failed to produce discrete L DNA transcripts.

. . . . Drs. I. Nur, E. Pascale, and A. V. Furano

Methylation of the CpG dinucleotide leads to its eventual conversion to TpG (by deamination of 5 MeC to T). Therefore, the persistence of the L DNA promoter as a CpG island, in spite of the fact that it is fully methylated in the rat genome, means that the L DNA promoter only recently became subjected to methylation and that it is a particularly good substrate for methylation. Since our in vitro studies showed that methylation is important for repressing L promoter activity, we are now examining the factors affecting methylation of the L DNA promoter in vivo. To do this, we are determining the methylation of the L DNA promoter fused to a reporter gene when the fusion is present either as an extrachromosomal element or integrated into the chromosome. We are also examining the interaction between the L DNA promoter and the SV40 promoter/enhancer region.

. . . . Drs. E. Valle and A. V. Furano

Comparison among the L elements of various species has shown the presence of conserved open reading frames (ORFs) within these elements. An intact copy of one of the ORFs (ORF I) immediately downstream of the rat L DNA promoter has been found and will be used to produce the encoded protein which can then be used to raise antibodies or to analyze its interactions with the L element.

Other studies in our laboratory have shown that the L DNA promoter is capable of activating heterologous genes, implying the existence of transcription factors which interact with the promoter. An investigation to map the site of action and to isolate these factors is underway.

. . . . Drs. B. E. Hayward and A. V. Furano

We recently isolated a rat genomic clone that contained two L DNA elements in tandem; a full length (6.7 kb) element was followed after about 2 kb of DNA by the beginning of a second L element. To understand why two L elements inserted into this region, we determined the sequence of the DNA that separated the two L DNA elements and found that this DNA contains a portion of an L element that is as related to the present day rat L element as it is to present day mouse L family. Therefore, it bears a similar ancestral relationship to both present day rodent L families, and hybridization experiments showed that L ranc (rodent ancestral) is present in about the same copy number in both rodent genomes. The evolutionary origin and fate of L DNA elements is completely unknown. In particular, such questions as whether ancestral mammalian organisms contained a high number of L elements and whether they were replaced during evolution by present day elements may be answered by our current studies on L ranc.

. . . . Drs. E. Pascale and A. V. Furano

We are studying the E. coli bacteriophage T4 as a model system for duplex DNA replication. Efficient DNA replication in vitro is achieved with seven purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43 product), gene 32 DNA helix-destabilizing protein, the gene 44/62 and gene 45 polymerase accessory proteins, and the genes 41 and 61 proteins which function together as a primase and as a DNA unwinding enzyme (or helicase).

Assembly of T4 DNA Polymerase and Its Accessory Proteins at the Primer-Terminus. Previous mechanistic studies of the effect of the accessory proteins on both the polymerase and exonuclease activities of T4 DNA polymerase support a model in which the accessory proteins function as a sliding clamp to keep the polymerase bound to the 3' OH-terminus. Our recent gel filtration binding studies indicate that this clamp is assembled stepwise. Polymerase and the 44/62 protein bind independently to primed ssDNA in the absence of ATP. Both proteins bind more tightly to DNA coated with 32 protein. Subsequent binding of 45 protein requires ATP as well as both the polymerase and the 44/62 protein complex. Isolated complexes of oligonucleotide-primed circular ssDNA with polymerase, 32 protein, and the three accessory proteins are capable of very rapid (200 n/sec) copying of the ssDNA.

Efficient Initiation of Strand Displacement Synthesis Requires a Forked DNA Template. We have used synthetic deoxyoligonucleotide to construct nicked and forked circular templates to determine the structural requirements for establishing leading strand synthesis by polymerase, the accessory proteins, and 32 protein. Strand displacement synthesis is initiated on only a small fraction of the nicked templates. In contrast, a 50 base region on the side of the fork corresponding to the lagging strand template allows synchronous and very rapid initiation of leading strand synthesis on all of the DNA molecules. This requirement for a preformed fork indicates that the polymerase and/or its accessory proteins is in contact with both template strands at the replication fork. The 50 base preformed fork also increases the fraction of the molecules initially synthesizing at the very high rate dependent on the 41 protein helicase. More importantly, on forked, but not on nicked, templates, there is 41 protein-dependent strand displacement synthesis on some molecules in the absence of 32 protein. These still preliminary results support a model in which 41 protein helicase controls unwinding of the duplex ahead of the leading strand, while 32 protein acts to prevent reannealing of the strands unwound.

. . . . Dr. N. G. Nossal

The C-Terminal Region of the 41 Primase-Helicase Protein Is Required for Its Interacting with Other Enzymes in the Replication Complex. A tryptic fragment of 41 protein which we call 41T, missing 17 or 20 amino acids from the C-terminus, retains the ssDNA-stimulated ATPase and the helicase activity of the intact enzyme. 41T also retains the ability to interact normally with 61 protein: 41T helicase is stimulated by 61 protein and 41T is fully competent in promoting pentamer primer synthesis by 61 protein. In contrast, 41T is severely impaired in its ability

to function in more complex reactions involving the other replication proteins.

In the absence of 32 ssDNA binding protein, the 41T/61 primase-helicase primes DNA synthesis on ssDNA templates as well as the intact enzyme, but, unlike the intact 41 protein, 41T cannot prime DNA synthesis on DNA covered with 32 protein. We find that high concentrations of 32 protein strongly inhibit primer synthesis with either 41T or the intact protein. The difference is that addition of the 44/62 and 45 polymerase accessory proteins substantially reverses the 32 protein inhibition of primer synthesis with intact 41 protein, but cannot reverse the inhibition with 41T. Similarly, the 41T helicase is active by itself or with 61 protein, but cannot stimulate strand displacement synthesis by the other T4 proteins.

We propose that 41 protein interacts with the T4 polymerase accessory proteins to facilitate primer synthesis and DNA unwinding on 32 protein-coated DNA. Alteration of the C-terminus in 41T protein prevents the assembly of a functional primase-helicase within the replication enzyme complex.

. . . . Drs. R. W. Richardson and N. G. Nossal

Bacteriophage T4 Gene Expression. The expression of a region of the bacteriophage T4 genome is being studied as a model for examining developmental gene regulation. Throughout infection, T4 uses the host transcriptional apparatus to transcribe its DNA, but with time different regions of the T4 genome are transcribed. To determine host and phage signals and factors that regulate this transcription, the expression of the T4 genes uvsX (recombination protein), 40 (stimulates head formation), and 41 (primase-helicase component) have been examined by nuclease S1 protection experiments and Northern analyses.

Changes in the transcription pattern of the uvsX-40-41 region are programmed with phage development. Early in infection a heterogeneous population of transcripts is observed, having major 5' starts approximately 900 and 200 bases upstream of uvsX. The bulk of these RNAs continue through 40 and into 41, but a portion (about 1/4) stop just downstream of uvsX (within gene 40). Later in infection a 5' start closer to uvsX (about 50 bases upstream) is observed, in addition to the ends seen earlier, and a significant fraction of the uvsX RNA ends at the stop. Thus, as infection proceeds, the level of 40,41 mRNA decreases relative to that of uvsX.

Analysis of the expression of the uvsX-40-41 region when cloned on a plasmid and present in an uninfected cell reveals different 5' ends from those present after T4 infection, but significant utilization of the 3' RNA stop downstream of uvsX. Thus, T4 infection is needed for the 5' starts, while the host system alone can produce the RNA stop. To help determine what host factor(s) are needed for this step, expression of the uvsX-40-41 region upon infection of the Escherichia coli transcription termination (rho) mutant, rho026, was examined. This mutant is a

nonpermissive host for T4 in which the levels of 41 and other T4 proteins are depressed [Stitt et al. (1980) *J. Virol.* 35, 775]. Previous work has suggested that in this mutant, transcription termination is somehow enhanced, resulting in less expression of regions downstream of a rho-dependent termination site. Transcription mapping indicates that the level of 40, 41 mRNA expressed after infection of rho026 is several-fold lower than that expressed in the rho' parent. The decrease is due both to a 2.5-fold greater use of the RNA stop and to less transcription overall. Thus, rho factor acts either directly to terminate transcription at the stop between uvsX and 41 or indirectly to influence other factors needed for the correct regulation of this site.

. . . . Dr. D. M. Hinton

Hepatitis Non-A, Non-B. Hepatitis non-A, non-B (HNANB) is a world-wide problem, and 90% of the transfusion-related hepatitis cases in the United States (and 80-90% in several other countries) are diagnosed as HNANB. Approximately 50% of all acute HNANB patients develop chronic HNANB (an estimate of 4 million persons). They remain as potential sources of infection. Recent publications suggest a correlation between certain hepatocellular carcinomas and chronic HNANB infections.

Based on biochemical, immunological, and morphological evidence, we suggested that the HNANB agent is a mammalian type C retrovirus. Recently, using an in vitro focus-induction assay developed for mammalian type C viruses, we observed that pelleted material from HNANB sera (transfusion-related) induced foci formation. This result is consistent with the presence of a mammalian type C virus in HNANB sera.

A DNA probe of 780 base pairs (based on agarose gel analysis) isolated from HNANB-infected chimpanzee liver and selected by subtractive hybridization with normal chimpanzee liver was shown to hybridize in situ with liver sections from three HNANB-infected chimpanzees but not with liver from two HBV-infected animals. This DNA fragment has been cloned, completely sequenced, and placed under the control of the Sp6 promoter. Sequence data indicated that the DNA fragment is 757 base pairs. The vector pBR322 and the 757 base fragment has been designated pSC22.

Cultures of peripheral blood mononuclear cells isolated from a HNANB patient (R.F.) was found to hybridize to the 757 base pair probe. Also, cultures of the mink lung line (CCL64.1) superinfected with HNANB viral particles (from 15 patients) hybridize specifically with the 757 base probe. These experiments are still in progress.

The DNA probe of 757 base pairs isolated from HNANB-infected chimpanzee liver and selected by subtractive hybridization with normal chimpanzee liver was shown to hybridize with liver from HNANB-infected chimpanzees. Hybridization of this DNA probe to HNANB-infected peripheral mononuclear

blood cultures and the mink lung cell superinfected with pelleted material from HANB-infected sera is being tested.

. . . . Drs. W. G. Coleman, Jr., A. W. Gordon, and L. Chen, and B. P. Seto (HL, DBBP, Center for Drugs and Biologics)

IV. MEMBRANE STUDIES OF MACROPHAGES AND OF ESCHERICHIA COLI

Aldoheptose Biosynthesis. Previously, a novobiocin-hypersensitive mutant of Escherichia coli K-12 carrying a cysE-pyrE linked mutation, designated rfaD, which specifically affects the synthesis of the aldoheptose, L-glycero-D-mannoheptose, has been isolated and genetically characterized. The rfaD gene codes for ADP-L-glycero-D-mannoheptose-6-epimerase, an enzyme required for lipopolysaccharide (LPS) core biosynthesis. The nucleotide ADP-D-glycero-D-mannoheptose accumulates in rfaD mutant strains. The rfaD phenotype includes increased permeability to a large number of hydrophobic drugs and dyes, and the formation of mucoid colonies. A 9-kilobase DNA EcoRI fragment carrying the rfaD gene was initially identified in the Clarke-Carbon Colony Bank cloned in pBR322, and subsequently smaller restriction fragments were cloned into several expression plasmid vectors. The proteins expressed by RfaD plasmids, using several in vivo and in vitro expression systems, have been examined by SDS gel electrophoresis. RfaD plasmids express a protein with a molecular weight of 37,000. One of these plasmids, pJP5, which contains a 1.8-kilobase EcoRI-NruI fragment, expresses the rfaD gene product and complements all of the phenotypes associated with the rfaD mutation. Finally ADP-L-glycero-D-mannoheptose-6-epimerase has been purified to homogeneity by 60% ammonium sulfate precipitation, followed by ion exchange and gel filtration. The gel filtration profile also indicates that the rfaD gene product has a molecular weight of 37,000. The 1.8-kilobase EcoRI-NruI fragment has been cloned into M13mp18 and M13mp19 and sequenced using the dideoxy sequence method.

. . . . Drs. J. C. Pegues and W. G. Coleman, Jr.

V. ENZYME MECHANISMS AND PROTEIN STRUCTURE

Three-Dimensional Structure of the Tryptophan Synthase $\alpha_2\beta_2$ Multienzyme Complex from Salmonella typhimurium. The three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ complex has been determined by x-ray crystallography at 2.5 Å resolution. The four polypeptide chains are arranged linearly in an $\alpha\beta\beta\alpha$ order, forming a complex 145 Å long. The active sites of the neighboring α and β subunits are 25 Å apart and are connected by a tunnel which facilitates the diffusion of indole from the active site of the α subunit to the active site of the β subunit. Microspectrophotometric studies of single crystals of tryptophan synthase in the presence and absence of ligands provide information for

future x-ray crystallographic studies in the presence of ligands which will identify active site residues in the β_2 subunit.

. Drs. C. C. Hyde (LMB, NIDDK), E. A. Padlan (LMB, NIDDK), S. A. Ahmed, E. W. Miles, D. R. Davies (LMB, NIDDK), A. Mozzarelli (University of Parma, Italy), G. L. Rossi (University of Parma, Italy), and M. F. Dunn (University of California, Riverside)

Site-Directed Mutagenesis of Tryptophan Synthase from Salmonella typhimurium. Site-directed mutagenesis of tryptophan synthase has been initiated in order to investigate the effects of structure on the functional properties of this multienzyme complex. Two mutant forms of the α subunit have been prepared in which either cysteine-81 or cysteine-118 is replaced by a serine residue. The fully active purified mutant forms of the $\alpha_2\beta_2$ complex are potentially useful for x-ray crystallographic studies since a heavy metal binding site is specifically eliminated in each mutant. Aspartic acid-60 of the α subunit has been replaced by four different amino acids. Studies of these mutants provide evidence that aspartic acid-60 is an essential catalytic base in reactions catalyzed by the α subunit. Studies of single and double mutants at tyrosine-175 and cysteine-211 show that these residues have important roles in substrate binding but are not essential for catalysis. The potential active site roles of a series of residues of the β subunit are being explored by replacing each of these residues by one or two amino acids. Mutant forms have been prepared at each of the following residues: His-86, Lys-87, Arg-148, Cys-170, Cys-230, Glu-109, Gln-114, His-115, Asp-305, and Glu-350. The mutant in which lysine-87, which normally forms a Schiff base with pyridoxal phosphate, is converted to threonine is especially interesting. This enzyme, although catalytically inactive, is able to form Schiff base intermediates with substrates. We are also attempting to design and isolate mutants in which the tunnel is blocked. All of the mutant forms of the $\alpha_2\beta_2$ complex specified above have been purified by a new efficient purification procedure which utilizes crystallization from crude extracts. Crystals of some of the mutant enzymes are being grown for x-ray crystallography.

. Drs. E. W. Miles, H. Kawasaki, S. A. Ahmed, R. Bauerle (University of Virginia, Charlottesville), S. Nagata, H. Morita, and H. Morita

Detection and Identification of Transient Intermediates in Reactions of Tryptophan Synthase with Reaction Intermediate Analogs. The pre-steady-state reactions of two reaction intermediate analogs (2,3-dihydro-L-tryptophan and oxindolyl-L-alanine) with tryptophan synthase have been investigated by rapid scanning stopped-flow spectrophotometry. The rates of formation of several intermediates, including a previously undetected gem-diamine intermediate, have been determined. These studies shed light on the mechanism of tryptophan synthase.

. Drs. E. W. Miles, M. Roy (University of California, Riverside), and M. F. Dunn (University of California, Riverside)

Evolution of Protein Folding. We have compared the kinetic and equilibrium unfolding properties of the α subunit of tryptophan synthase from *Escherichia coli*, *Salmonella typhimurium*, and five interspecies hybrids. The results show that all the proteins follow the same folding mechanism and support the hypothesis that the folding mechanism is conserved in homologous proteins.

. . . . Drs. E. W. Miles and C. R. Matthews (Pennsylvania State University, University Park)

A novel experimental method for rapid qualitative characterization of heterogeneous mixtures of macromolecules has been proposed, based upon analysis of the time and concentration dependence of two experimentally measurable parameters called the apparent sedimentation and diffusion coefficients.

. . . . Drs. R. C. Chatelier and A. P. Minton

A simple method for reducing the duration of a conventional sedimentation equilibrium by a factor of approximately five has been proposed.

. . . . Dr. R. C. Chatelier

The interaction between fibrous rabbit muscle actin and several globular proteins has been investigated via size exclusion chromatography, sedimentation, and viscosity measurement.

. . . . Drs. S. Lakatos and R. C. Chatelier

A PC-based data acquisition and control system for the UV-visible scanner on the Beckman Model E analytical centrifuge has been developed and installed.

. . . . Drs. A. P. Minton and M. S. Lewis (BEIB, DRS)

An approximate theory has been formulated for the dependence of the diffusion coefficient of each species of protein upon the concentrations of all species in a mixture of globular proteins.

. . . . Dr. A. P. Minton

A most important recent finding is that activation of a complex-bound valyl-tRNA synthetase requires tRNA, and that a heat-stable regulatory protein is associated with tRNA. It appears that the oxido-reductive regulatory function of the protein's thiol groups is transmitted through an effect on tRNA conformation. The nucleic acid-protein complex is obtained from the soluble portion of a heated extract by precipitation in acid. The protein can be obtained from the complex by extraction into phenol, which is then removed by dialysis against dilute hydro-

chloric acid (pH 3). The acid-soluble protein becomes insoluble when neutralized but dissolves in a neutral solution of tRNA.

. Dr. S. Black

Cooperative binding systems are being studied taking into account site or subunit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems.

Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and homology of these sequences. The property of uniqueness (the occurrence of a small peptide at a frequency considerably less than that expected) has been quantified, and speculations on this quantity and the immune response are under investigation.

. Drs. H. A. Saroff and E. Mihalyi

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 23,140-30 LBP

PERIOD COVERED

January 1, 1988 through September 30, 1988

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

Biochemistry of Sulfur-Containing Compounds

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

PI: Simon Black, Ph.D. Chemist (Research)

LBP NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.3

PROFESSIONAL

0.8

OTHER

0.5

CHECK APPROPRIATE BOX(ES)

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

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

A most important recent finding is that activation of a complex-bound valyl-tRNA synthetase requires tRNA, and that a heat-stable regulatory protein is associated with tRNA. It appears that the oxido-reductive regulatory function of the protein's thiol groups is transmitted through an effect on tRNA conformation. The nucleic acid-protein complex is obtained from the soluble portion of a heated extract by precipitation in acid. The protein can be obtained from the complex by extraction into phenol, which is then removed by dialysis against dilute hydrochloric acid (pH 3). The acid-soluble protein becomes insoluble when neutralized but dissolves in a neutral solution of tRNA.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 23,330-10 LBP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Aldoheptose Biosynthesis and Its Regulation

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

PI: William G. Coleman, Jr., Ph.D. Research Microbiologist LBP NIDDK

Others: Alfred W. Gordon, Ph.D. IRTA LBP NIDDK
 Joyce C. Pegues, Ph.D. Staff Fellow LBP NIDDK
 Lishi Chen, Ph.D. Visiting Fellow LBP NIDDK
 Dong Hyun Chung, M.D., Ph.D. Special Volunteer LBP NIDDK

COOPERATING UNITS (if any)

Belinda P. Seto, Ph.D., HL, DBBP, Center for Biologics, Research, and Review

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5.3

PROFESSIONAL:

5.0

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

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

Aldoheptose Biosynthesis. Previously, a novobiocin-hypersensitive mutant of *Escherichia coli* K-12 carrying a *cysE-pyrE* linked mutation, designated *rfaD*, which specifically affects the synthesis of the aldoheptose, L-glycero-D-mannoheptose, has been isolated and genetically characterized. The *rfaD* gene codes for ADP-L-glycero-D-mannoheptose-6-epimerase, an enzyme required for lipopolysaccharide (LPS) core biosynthesis. The nucleotide ADP-D-glycero-D-mannoheptose accumulates in *rfaD* mutant strains. The *rfaD* phenotype includes increased permeability to a large number of hydrophobic antibiotics, and the formation of mucoid colonies. A 9-kilo-base DNA *EcoRI* fragment carrying the *rfaD* gene was initially identified in the Clarke-Carbon Colony Bank cloned in pBR322, and subsequently smaller restriction fragments were cloned into several expression plasmid vectors. *RfaD*⁺ plasmids express a protein with a molecular weight of 37,000, and all complement all phenotypes associated with the *rfaD* mutation. Finally ADP-L-glycero-D-mannoheptose-6-epimerase has been purified to homogeneity. The entire sequence encoding the *rfaD* product has been sequenced using the dideoxy sequencing method.

Hepatitis Non-A, Non-B. Hepatitis non-A, non-B (HNANB) is a world-wide problem, and 90% of the transfusion-related hepatitis cases in the United States (and 80-90% in several other countries) are diagnosed (by exclusion) as HNANB. Approximately 50% of all acute HNANB patients develop chronic HNANB.

Previously we used an *in vitro* focus-induction assay developed for mammalian type C retroviruses, and we observed that pelleted material from HNANB sera (transfusion-related) induced foci formation.

The DNA probe of 757 base pairs isolated from HNANB-infected chimpanzee liver and selected by subtractive hybridization with normal chimpanzee liver was shown to hybridize with liver from HNANB-infected chimpanzees. Hybridization of this DNA probe to HNANB-infected peripheral mononuclear blood cultures and the mink lung cell superinfected with pelleted material from HNANB-infected sera is being tested.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 23,580-25 LBP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Mammalian Transposons

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

PI: Anthony V. Furano, M.D. Medical Officer (Research)
and Chief, Section on Genomic Structure and Function, LBP LBP NIDDK

Others: Bruce E. Hayward, Ph.D. Visiting Fellow LBP NIDDK
Esterina Pascale, Ph.D. Visiting Fellow LBP NIDDK
Karen Usdin, Ph.D. Visiting Fellow LBP NIDDK
Eulalia Valle, Ph.D. Special Volunteer LBP NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Genomic Structure and Function

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.5

PROFESSIONAL:

4.3

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

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

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

Members of the L transposon family (long interspersed repeat DNA or LINE family) of rats are 6.7 kb long, 5 kb of which is devoted to protein encoding sequence (ORFs). A promoter-like sequence is at the left end of the element, and G-rich homopurine (GHP) stretches are at the other end. Although 5 kb or so of the rat and mouse L DNA is very highly conserved, their promoter-like sequences are completely distinct. This means that novel species-specific promoter sequences have been repeatedly acquired during the evolution of L families. The rat promoter sequence contains enough CpGs to qualify it as a "CG island" and we have recently shown that this sequence functions as a promoter in vivo since it activates the prokaryotic chloramphenicol acyltransferase gene in rat cells. Furthermore, partial methylation of the promoter inhibits its activity by about 75% in these cells. All mammalian L elements contain at their right end GHP stretches. We recently showed that these sequences induce unpairing of contiguous duplex DNA such that this DNA can take up (hybridize) complementary DNA sequences. Furthermore, the hybridized DNA sequence can be elongated by added DNA polymerase. Both of these phenomena are essential intermediates in well-documented models for certain types of recombinational and transpositional events. This suggests that the L element GHP stretches may be very important for these properties of L DNA. Analysis of the structure of the GHP sequence itself, with a variety of chemical and enzymatic probes, showed that this sequence does not assume any stable non-B DNA basepaired structure, but rather exhibits significant single-stranded character. Therefore, the GHP stretch is functionally unpaired, and this property provides a straightforward explanation for the effect of GHP stretches on contiguous duplex DNA. Finally, we have identified a novel L DNA element in the rat genome that appears to be the ancestral L element from which both present day rat and mouse L families were derived. Study of this ancestral L element should clarify the evolutionary origin and fate of L DNA.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 23,750-02 LBP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Bacteriophage T4 Gene Expression

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

PI: Deborah M. Hinton, Ph.D.

Research Chemist

LBP NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Nucleic Acid Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.2

PROFESSIONAL

1.0

OTHER

0.2

CHECK APPROPRIATE BOX(ES)

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

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

The expression of a region of the bacteriophage T4 genome is being studied as a model for examining developmental gene regulation. Throughout infection, T4 uses the host transcriptional apparatus to transcribe its DNA, but with time different regions of the T4 genome are transcribed. To determine signals and factors that regulate this transcription, the expression of the T4 genes uvsX (recombination protein), 40 (stimulates head formation), and 41 (primase-helicase component) have been examined by nuclease S1 protection experiments and Northern analyses.

Changes in the transcription pattern of the uvsX-40-41 region are programmed with phage development. Early in infection a heterogeneous population of transcripts is observed, having major 5' starts about 900 and 200 bases upstream of uvsX. The bulk of these RNAs continue through 40 and into 41, but a portion (about 1/4) stop just downstream of uvsX (within gene 40). Later in infection a 5' start closer to uvsX (about 50 bases upstream) is observed, in addition to the ends seen earlier, and a significant fraction of the uvsX RNA ends at the stop. Thus, as infection proceeds, the level of 40, 41 mRNA decreases relative to that of uvsX.

Analysis of uvsX-40-41 transcripts expressed by a plasmid with this region reveals different 5' ends from those after T4 infection, but significant utilization of the RNA stop downstream of uvsX. Thus, T4 infection is needed for the 5' starts, while the host can produce the RNA stop. To help determine what host factor(s) are needed for this stop, transcripts were examined after infection of the *E. coli* transcription termination (ρ) mutant, ρ 026. Previous work has suggested that in ρ 026, transcription termination may be enhanced, resulting in less expression of regions downstream of a ρ -dependent termination site [Stitt et al. (1980) *J. Virol.* **35**, 775]. My transcription mapping indicates that infection of ρ 026 results in several-fold less 40,41 mRNA than infection of the ρ + parent, because of a 2.5-fold greater use of the RNA stop and less transcription overall. Thus, ρ factor either acts to terminate transcription at the stop or influences other factors needed for the regulation of this site.



NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,140-22 LBP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Tryptophan Synthase: Structure and Function and Relationship to Tryptophanase

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

PI:	Edith Wilson Miles, Ph.D.	Research Chemist	LBP NIDDK
Others:	Shinji Nagata, Ph.D.	Visiting Fellow	LBP NIDDK
	Hiroshi Kanzaki, Ph.D.	Visiting Fellow	LBP NIDDK
	Yoshihiro Sawa, Ph.D.	Visiting Fellow	LBP NIDDK

COOPERATING UNITS (if any) Drs. D. R. Davies, C. C. Hyde, and E. A. Padlan, LMB, NIDDK; R. Bauerle, Univ. of Virginia, Charlottesville; M. Roy and M. F. Dunn, Univ. of California, Riverside; C. R. Matthews, Pennsylvania State Univ., University Park; A. Mozzarelli and G. L. Rossi, Univ. of Parma, Italy; K. Yutani, Osaka Univ., Japan

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.7

PROFESSIONAL

2.4

OTHER

0.3

CHECK APPROPRIATE BOX(ES)

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

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

We are investigating the structure and function of the bacterial tryptophan synthase $\alpha\beta 2$ complex by use of x-ray crystallography, site-directed mutagenesis, and spectroscopic studies. The tryptophan synthase multienzyme complex catalyzes the final reaction in L-tryptophan biosynthesis and has been the subject of many genetic and biochemical studies. Our recent determination of the three-dimensional structure of this multienzyme complex by x-ray crystallography allows us to locate the active sites of both the α and β subunits and a tunnel which connects these two sites. The 25 A tunnel facilitates the diffusion of indole produced at the active site of the α subunit to the active site of the β subunit. Site-directed mutagenesis of tryptophan synthase has been initiated in order to investigate the effects of structure on the functional properties of tryptophan synthase. We have designed and prepared a series of mutant forms of the α and β subunit by site-directed mutagenesis. More than 30 such mutant proteins have been isolated by a new efficient purification procedure in which the $\alpha\beta 2$ complex is crystallized from a crude extract. Two mutants in which either cysteine-81 or cysteine-118 of the α subunit is replaced by a serine residue are potentially useful for x-ray crystallographic studies, since a heavy metal binding site is specifically eliminated in each mutant. Studies of four mutants in which aspartic acid-60 of the α subunit was changed to four different amino acids indicate that aspartic acid-60 is an essential catalytic base. Studies of single and double mutants at tyrosine-175 and cysteine-211 of the α subunit support a substrate binding role for these residues. Studies of mutations at several sites in the $\beta 2$ subunit are defining the active site roles of these residues. We are also trying to produce mutations which block the tunnel which extends between the active sites of the α and β subunits. Spectroscopic methods have been used to study transient reaction intermediates with substrate analogs and to study the kinetics of folding and unfolding of the α subunit.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 24,150-17 LBP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Noncovalent Intermolecular Interactions in Biochemistry

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

PI: Allen P. Minton, Ph.D. Research Chemist LBP NIDDK
Others: Ronald C. Chatelier, Ph.D. Visiting Fellow LBP NIDDK
Susan Lakatos, Ph.D. Visiting Fellow LBP NIDDK

COOPERATING UNITS (if any)

M. S. Lewis, Biomedical Engineering and Instrumentation Branch, Division of Research Services, NIH; M. Sokolovsky, Tel Aviv University, Tel Aviv, Israel

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

3.5

PROFESSIONAL

3.3

OTHER

0.2

CHECK APPROPRIATE BOX(ES)

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

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

A novel experimental method for rapid qualitative characterization of heterogeneous mixtures of macrosolutes has been proposed, based upon analysis of the time and concentration dependence of two experimentally measurable parameters called the apparent sedimentation and diffusion coefficients.

A simple method for reducing the duration of a conventional sedimentation equilibrium by a factor of approximately five has been proposed.

The interaction between fibrous rabbit muscle actin and several globular proteins has been investigated via size exclusion chromatography, sedimentation, and viscosity measurement.

A PC-based data acquisition and control system for the UV-visible scanner on the Beckman Model E analytical centrifuge has been developed and installed.

An approximate theory has been formulated for the dependence of the diffusion coefficient of each species of protein upon the concentrations of all species in a mixture of globular proteins.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,260-22 LBP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Enzymatic Mechanisms of DNA Replication: The Bacteriophage T4 System

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

PI: Nancy G. Nossal, Ph.D. Research Chemist and Chief,
Section on Nucleic Acid Biochemistry, LBP LBP NIDDK

Other: Ross W. Richardson, Ph.D. Staff Fellow LBP NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Nucleic Acid Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.3

PROFESSIONAL

2.0

OTHER

0.3

CHECK APPROPRIATE BOXES)

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

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

In the *E. coli* bacteriophage T4 model system for duplex DNA replication, efficient DNA replication in vitro is achieved with seven purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43 product), gene 32 DNA helix-destabilizing protein, the gene 44/62 and gene 45 polymerase accessory proteins, and the genes 41 and 61 proteins, which together function as a primase and a helicase.

Polymerase-Accessory Protein Interactions. The three accessory proteins function as a sliding clamp to keep the polymerase bound to the primer-template. Polymerase and the 44/62 protein bind independently to oligonucleotide-primed ssDNA in the absence of ATP. Subsequent binding of 45 protein requires ATP as well as both polymerase and the 44/62 protein complex.

Strand Displacement Synthesis on Forked Templates. A 50 base region on the side of a synthetic forked DNA substrate corresponding to the lagging strand template allows synchronous and rapid interaction of leading strand displacement synthesis, indicating that the leading strand polymerase and/or its accessory proteins is in contact with both template strands at the replication fork. The preformed fork also permits 41 protein-dependent strand displacement synthesis on some molecules in the absence of 32 protein. These results are consistent with a model in which 41 protein helicase controls unwinding of the duplex, while 32 protein acts to prevent reannealing of the strands unwound.

Primase-Helicase Interactions with Other Replication Proteins. A tryptic fragment of 41 protein, missing 17 or 20 amino acids from the C-terminus, retains the primase and helicase activities of the intact enzyme, but is unable to catalyze these activities in the presence of the other replication proteins. Primer synthesis by 61 protein, and either intact 41 protein or the tryptic fragment, is inhibited by high concentrations of 32 protein. The polymerase accessory proteins reverse this inhibition with intact 41 protein but not with the 41 fragment. Thus, alteration of the C-terminus of 41 protein prevents an interaction with the accessory proteins which is necessary for primer synthesis on 32 protein coated DNA.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 24,590-17 LBP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Structure and Interactions of Biologically Important Macromolecules

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

PI: Harry A. Saroff, Ph.D. Research Chemist (Intermittent) LBP NIDDK
and Scientist Emeritus

Other: Elemer Mihalyi, M.D., Ph.D. Special Volunteer LBP NIDDK

COOPERATING UNITS (if any)

A. Patchornik, Weizmann Institute of Science, Rehovot, Israel
National Center for Drugs and Biologics

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.1

PROFESSIONAL

2.0

OTHER

0.1

CHECK APPROPRIATE BOXES)

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

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

Cooperative binding systems are being studied taking into account site or subunit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems.

Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and homology of these sequences. The property of uniqueness (the occurrence of a small peptide at a frequency considerably less than that expected) has been quantified, and speculations on this quantity and the immune response are under investigation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,709-07 LBP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Polyamine Biosynthesis and Function

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

PI: Celia White Tabor, M.D. Medical Director, USPHS LBP NIDDK

Others: Herbert Tabor, M.D. Supervisory Medical Officer
(Research); Chief, Section on Pharmacology, LBP; and
Chief, Laboratory of Biochemical Pharmacology LBP NIDDK
Qiao-Wen Xie, Ph.D. Visiting Associate LBP NIDDK
Keiko Kashiwagi, Ph.D. Visiting Fellow LBP NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Pharmacology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.8

PROFESSIONAL:

3.7

OTHER:

1.1

CHECK APPROPRIATE BOXES)

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

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

Polyamines (such as putrescine, spermidine, and spermine) are major cellular components, and have been shown to be involved in many systems related to growth and differentiation. Our studies have been directed at learning how these polyamines are synthesized and regulated, and their physiological function. To this end we have carried out a wide variety of genetic and biochemical studies. We have: (1) established the pathways for the biosynthesis of these amines; (2) isolated the enzymes involved; (3) identified the genes responsible for each of these steps and constructed mutants lacking the encoded enzymes; (4) constructed plasmids that contain these genes, and that permit overproduction of the various enzymes; (5) studied the physiological effects of amine deprivation in vivo on ribosome action and on protein biosynthesis; (6) shown that the genes coding for spermidine synthase (speE) for S-adenosylmethionine decarboxylase (speD) form an operon at 2.7 minutes on the Escherichia coli chromosome. We have sequenced and characterized this operon. (7) We have shown that S-adenosylmethionine decarboxylase is formed as proenzyme which is then processed by a post-translational cleavage at a lysyl-serine peptide to form two subunits, one of which contains the pyruvoyl group that is found in the mature enzyme and is essential for enzymatic activity. Mutants in which other amino acids are substituted for the lysine result in much slower processing. (8) We have carried out comparable studies in Saccharomyces cerevisiae.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,940-15 LBP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Yeast RNA Virology

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

PI:	Reed B. Wickner, M.D.	Medical Director, USPHS, and Chief, Section on Genetics of Simple Eukaryotes, LBP	LBP NIDDK
Others:	Yutaka Matsumoto, Ph.D.	IRTA	LBP NIDDK
	Tsutoma Fujimura, Ph.D.	Visiting Associate	LBP NIDDK
	Tateo Icho, Ph.D.	Visiting Associate	LBP NIDDK
	Sang Ki Rhee, Ph.D.	Visiting Associate	LBP NIDDK
	M. Rosa Canibano Esteban, Ph.D.	Visiting Fellow	LBP NIDDK
	Yang-Ja Lee, Ph.D.	Special Volunteer	LBP NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Genetics of Simple Eukaryotes

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

6.0

PROFESSIONAL

5.7

OTHER

0.3

CHECK APPROPRIATE BOX(ES)

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

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

There are five families of double-stranded RNA virus-like particles (L-A, L-BC, M, T, and W) and two distinct single-stranded RNA virus-like entities (20S RNA and 23S RNA) that replicate in cells of Saccharomyces cerevisiae. We have studied how these genomes replicate in yeast with emphasis on the role of the host. Highly purified virus-like particles (VLPs) carry out both (+) strand and (-) strand synthesis of L-A, L-BC, or M RNA in vitro in a conservative, sequential reaction.

We developed an in vitro replication system [(-) strand synthesis on a viral (+) strand template producing dsRNA] for the L-A system. Using isolated opened empty viral particles, we demonstrated site-specific binding of viral (+) strands. The internal sequence 5' UUUGGCCAGG 3' determines this binding. In addition to this sequence, the replicate reaction requires the correct 3' end, but not the 5' end. The viral 180 kDa minor protein binds ssRNA and is encoded by L-A, having an N-terminal domain with immunologic relationship to the major coat protein and the C-terminal region responsible for ssRNA binding. We have cloned and sequenced the L-A genome and find that the 180 kDa protein is encoded by the fusion of two overlapping ORFs, ORF1 and ORF2. ORF1 encodes the major coat protein, and ORF2 encodes the ssRNA binding domain of the 180 kDa protein. ORF2 has substantial homology with viral RNA polymerases of picornaviruses and togaviruses. We have designed an RNA virus-based vector for yeast using our clone of X dsRNA, a deletion derivative of L-A dsRNA.

LABORATORY OF CHEMICAL BIOLOGY
NATIONAL INSTITUTE OF DIABETES, AND DIGESTIVE AND KIDNEY DISEASES
ANNUAL REPORT: October 1, 1987 to September 30, 1988

The Laboratory of Chemical Biology conducts research on structure, function and dynamics of proteins and on molecular biology and genetics, especially as related to genetic diseases. The Laboratory has several major foci of research. A major emphasis is in the identification of cis-acting regions in the DNA close to various human globin genes that may be involved in transcriptional control and on identifying the trans-acting proteins which bind to these DNA sequences and effect functional changes. A second major emphasis of the Laboratory is in the study of forces that stabilize globular proteins and also that contribute to the interaction of these proteins with antibodies. A third area is the study of the human T-cell receptor, especially its δ chain, and the genetic mechanism that lead to expression of receptors composed of α/β or δ/γ chains. A program on isolating and characterizing the tat protein of the HIV virus has also been initiated under sponsorship of the NIH Intramural AIDS Research Program which is specifically targeted to drug development. Other active projects include the clinical evaluation of sickle cell patients being treated with hydroxyurea to increase fetal hemoglobin levels, the development of a transgenic model of sickle cell anemia in the mouse and cytogenetic studies of a variety of human disease syndromes.

The reorganization of the Laboratory started several years ago is now largely complete. There are three sections. The Section on Protein Chemistry and Conformation, under Dr. Hiroshi Taniuchi, is devoted primarily to studying the folding and assembly of globular proteins, especially cytochrome c. The Section on Molecular Forces and Assembly is the new home of the Cytogenetics Unit under Dr. Beverly White, which is a joint endeavor of the Inter-Institute Genetics Program of the Clinical Center and NIDDK. The Section on Molecular Biology and Genetics, under Dr. Alan N. Schechter, is concerned primarily with the molecular basis of the developmental control of gene expression, especially in human erythroid and lymphoid cells, and its relevance to the understanding of the molecular basis of disease states and possible approaches to their therapy. Among new joint programs with other institutes in the Laboratory, in addition to the Cytogenetics Unit, are the AIDS project, studies of erythropoietin, and therapeutic protocols in sickle cell disease. These are detailed below.

During the last year there have been few major personnel changes. Dr. Beverly White's transfer to this Laboratory to establish a Cytogenetic Unit has been completed. Dr. David Cohen has become an Expert Consultant to develop his research efforts in the molecular genetics of normal and abnormal lymphoid cells. Dr. Griffin Rodgers completed four years as a Robert Wood Johnson Fellow and is now taking training at George Washington University in Hematology and Oncology, prior to returning to this Labora-

tory as a Senior Medical Staff Fellow. Dr. Donald Rau has transferred to the Laboratory of Biochemistry and Metabolism of NIDDK. Dr. C.B. Anfinsen continues as a Scientist Emeritus in this Laboratory and makes frequent visits here.

Extensive research collaborations exist within this Laboratory and with other Laboratories in this Institute, in NIH, and nationally and internationally as outlined in the individual Research Project Reports. A formal collaboration has been established with the Clinical Center's Inter-Institute Medical Genetics Program to fund a clinical and research cytogenetic program. Clinical collaborations also exist with the Clinical Hematology Branch of NHLBI and other units. It is under the aegis of this collaboration that the clinical studies of hydroxyurea treatment of sickle cell patients is done. The development of transgenic sickle cell mice is being done with the Metabolic and Developmental Neurology Branch of NINCDS. In addition, a formal collaboration has been established involving the exchange of personnel and resources with Dr. David Hankins of the Laboratory of Experimental Hematology of the Armed Forces Radiobiological Research Institute at the National Naval Medical Center. The participation of this Laboratory in the NIH Inter-Institute Medical Genetics Program and the NIH-George Washington University Hematology Training Program continues to grow. The Laboratory is now also a major part of the recently established Intramural AIDS Research Program. The work in this program involves collaborations with the Laboratory of Molecular Virology of NCI and KabiGen AB of Stockholm, Sweden. Other collaborations include those with the Inserm Unit at Hopital Henri Mondor at Creteil, France (including an exchange of personnel); the Division of Hematology at the University of Birmingham School of Medicine in England and the MRC Unit in Kingston, Jamaica.

Section on Protein Chemistry and Conformation

A new hypothesis for protein folding has been developed during the last several years. This is based on the studies of fragment complexes of cytochrome *c*, in particular the stability of various heterologous complexes and ones chemically synthesized with changed amino acids and of the binding of various monoclonal antibodies to cytochrome *c*. Originally these studies were interpreted generally in terms of globally coupling forces. But now they are more specifically being studied as a manifestation of a small number of closed loops for each protein that mediate the stability of the folded forms. For cytochrome *c* it is postulated that there are four such loops controlling stability. Analogously, the extreme sensitivity of antigen-antibody interactions to single amino acid changes, such as a change in binding constant of 10,000 following a substitution of glutamic acid 93 by alanine in cytochrome *c*, is also explained in terms of interatomic interactions of the closed loop - type between the protein and the antibody. This analysis of protein folding and stability is a new way of explaining perplexing problems remaining in more classical approaches.

Among the techniques being used in these studies are fragment complex exchange measurements; chemical synthesis; and production and characterization of monoclonal antibodies. A new state of the art amino acid analyzer has added a major instrument resource to the work of this Section.

Section on Molecular Forces and Assembly (Cytogenetics Unit)

This Unit has completed cytogenetic analyses on over 200 patients participating in NIH clinical protocols during the last year. The study of the correlation between nucleolus organizing regions (NOR) and Alzheimers disease has been completed with largely negative results, as has been the case for an examination of a possible correlation between schizophrenia and the fragile X chromosome syndrome. Several other clinical studies are still underway as is work to bring new techniques into the Unit especially in situ hybridization with cloned gene probes.

Section on Molecular Biology and Genetics

The major part of this Section's work is devoted to clarifying the molecular genetic basis by which the developmental switch from embryonic to fetal to adult hemoglobins occurs in the human. Understanding of the control of globin gene expression would be a very important general point with respect to developmental biology, but might also have specific therapeutic relevance for the diseases of hemoglobin. The project is being pursued for the most part by trying to understand the phenotype of a cell line, the K562 cells, which appears to be arrested in the late embryonic stage of globin gene expression. Evidence has been obtained that there are intranuclear factors, trans-acting factors, that determine which genes are expressed and which are silent in these cells. During the last year, a broad range program to identify and isolate these factors and to understand their mechanism of action has been developed. To this end studies are underway of the structure and function of the globin promoter regions (cis-acting sequences) by fusing families of deletion mutants to the gene for the enzyme chloramphenicol transferase (CAT) and assaying CAT activity in cells transfected with various promoter-CAT fusion genes. In addition direct binding assays (footprinting and gel shift) and subtractive cloning techniques are being used in order to isolate the protein or the gene for one or more of these trans-acting factors. During the last year these techniques have been used to identify several cis-sequences and several proteins (trans-acting) factors that interact in the 5' region of the human β - and ϵ -globin gene. The functional significance of each of these is now being investigated. In addition an in vitro transcription system has been developed which is specific for different globin genes and several genes have been cloned from induced K562 cells which may be involved in the induction phenomenon. Although the goals of characterizing these factors are not simple, the elucidation of the control of this biologically and medically important human gene system would be a potentially major step in molecular and developmental biology and in applied medical molecular genetics.

This Section also continues its work on the pathophysiology of sickle cell anemia. During the last year the role of fetal hemoglobin levels in determining disease severity and expected response to therapy has been clarified. A project to develop an animal model of sickle cell disease by using embryonal carcinoma cells and transgenic methods to introduce the β^S and the human α gene into mice has been initiated. Methods to remove the endogenous mouse globins, including site specific recombination, are also being studied. This work is regarded as a long term project to develop a true model of the disease for study of sickle cell rheology, pathophysiology, and treatment. A Clinical Center program to treat select patients

with hydroxyurea has been initiated and ten patients have been treated with significant elevations in fetal hemoglobin levels. Modified protocols using erythropoietin as well as hydroxyurea are being initiated.

Another program in the Section is the study of genes in human lymphoid tissues that code for the T-cell receptor (TCR), including the α , β , γ and δ genes. A new genetic element, TEA, has been identified in early human T cells and has been used to clone the δ gene. More detailed mapping studies during the last year have shown that the δ genes are nested among the α genes and that there may be a fairly novel genetic mechanism used for deletion of the δ gene during maturation to the adult α/β type T-cell receptor. This mechanism shares common features with class switching and Kappa gene deletion. Further, recent studies of lymphoid tumors suggest a temporal sequence of gene rearrangement and expression determines whether a T cell has an α/β or δ/γ type receptor. Transgenic methods are now being used to establish possible functions for the δ/γ receptor.

The NIH Intramural Research Program on AIDS has established a program in this Section to analyze transcriptional mechanisms related to the tat gene of HIV. The cloned tat gene has been obtained and has been inserted into an expression vector for large scale production of the tat protein in E. coli and eukaryotic cells. Several batches of the protein have been produced and partially purified from E. coli by KabiGen of Stockholm, Sweden and are now being further purified and characterized in this laboratory as is material made in insect cells with a baculo virus vector. The protein will be purified to allow detailed structure function studies, including X-ray crystallography and high resolution NMR. The tat gene is also being transfected into heterologous cells to examine its interaction with other promoters so as to clarify its molecular mechanism of action. We are trying to develop structural (binding) and functional assays to allow systematic study of potential inhibitors of tat function. We hope these studies may lead to a new approach to the treatment of AIDS.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25008-25 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

New Delocalized Interaction That Exists in Proteins and Controls Folding

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

PI:	Hiroshi Taniuchi,	Chief, Section on Protein Chemistry and Conformation	LCB, NIDDK
Other:	Alice Fisher	Chemist	LCB, NIDDK
	Xuan Truong	Biological Aid	LCB, NIDDK

COOPERATING UNITS (if any)

University of Padova, Padova, Italy

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry and Conformation

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

1.8

PROFESSIONAL:

1.3

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

In the previous years we have shown that RNase-(1-118) lacking 6 carboxy terminal residues of RNase A is not of lowest Gibbs free energy and highly motile despite the native like structure including 66% of the helical structure. We argue that if the conventional pair-wise interactions were to collectively account for the energy state of RNase-(1-118) the derivative should have been of lowest free energy. Thus, to explain the observations we hypothesize that some delocalized interaction exists in native RNase A to stabilize the structure. We refer to this new interaction as closed loop interaction assuming that a closed loop consisting of contacting groups would mediate delocalized interaction. Using the fragment complex system of cytochrome c developed in this Section, we wish to identify the hypothetical closed loop or loops that controls folding of cytochrome c. We have investigated hybrid complexes formed from heme- and apo-fragments (or apoproteins) of horse, tuna, candida and yeast iso-1-cytochrome c. The fragments of these species are found to be completely exchangeable with the following exception. Combination of tuna heme fragment (1-38)H and candida apofragment (61-109), but not that of candida heme (1-44)H and tuna apofragment (39-104), forms a complex with drastically decreased binding force. Hybrid complexes containing yeast apofragment (44-108) were not investigated. Comparing the amino acid sequences it is proposed that substitutions of leucine 9 (horse numbering) with threonine and leucine 98 with methionine are responsible for the weak binding of tuna heme-candida apofragment complex. This and other observations taken together with those previous have allowed us to assign the locations of four closed loops 1,2,3 and 4 to be above, at the right and the left sides of, and below the heme, respectively. Further, the previously found initial second order kinetic phase and the following first order phase are assigned to represent formations of closed loops 1 and 3, respectively. Loop 4 is presumed to have been added during evolution of eukaryotic cytochrome c from prokaryotic species.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Principles That Govern Protein Folding: Interaction Between Closed Loops

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

PI: Hiroshi Taniuchi Chief, Section on Protein Chemistry and Conformation LCB, NIDDK

Others: Boleslaw Picur Visiting Fellow LCB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry and Conformation

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

1.1

PROFESSIONAL:

1.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

The studies in this Section have led to the hypothesis that in proteins exists some new delocalized interaction that is mediated by a closed loop of contacting groups formed after folding and generates extra energy to stabilize the structure. As described in another report A. Fisher and H. Taniuchi have located four hypothetical closed loops in cytochrome c. Of these four, closed loop 1 is located above the heme and forms first in fragment complexation, closed loop 2 located at the left side of the heme is assumed to order residues 28 to 38 and closed loop 3 located at the right side to stabilize the Met 80-S-Fe bond. The previous studies have indicated that closed loop 2 interacts with closed loop 3. To analyze thermodynamics of this closed loop 2-closed loop 3 interaction we use the three fragment complex ferro(1-25)H•(28-38)•(39-104) and a fragment exchange technique. The previous studies have shown that in the presence of excess of fragment (28-38) it is possible to measure the rate of direct dissociation of fragment (39-104) i.e. without going through two fragment complex (1-25)H•(39-104). On the basis of this principle we plan to measure the effect of substitution of leucine 32 and leucine 35 with norvaline (one at a time) on the binding force of fragment (39-104). Thus, we have prepared the heme- and apo-fragments and radiolabelled (39-104) determined the dissociation rate of complex ferro(1-25)H•(39-104) as a function of temperature, resulting in activation Gibbs energy, 18.25 kcal/mol at 25 degrees C; activation enthalpy, 52.8 within 5.6 kcal/mol; and activation entropy, 116 within 20eu. This combined with the previous data suggest that reduction of heme strengthens the interaction of closed loop 1 or closed loop 3 or both. Using this information and the previous data of dissociation constants, measurements of the dissociation rate of fragment (39-104) from complex ferro-(1-25)H•(28-38)•(39-104) as a function of concentration of free fragment (28-38) should allow us to determine the rate constant of the direct dissociation. The same procedure will be used for the complex containing substitution at position 32 or 35.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25016-15 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Trans-Acting Factor(s) Controlling Globin Gene Expression in K562 Cells

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

PI:	Pablo Gutman	Visiting Fellow	LCB, NIDDK
Others:	Shi-Xian Cao	Visiting Associate	LCB, NIDDK
	Harish Dave	Visiting Fellow	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

1.1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

K562 is an erythroleukemic cell line used as a model for the study of the control of human globin gene expression. These cells do not support transcription of the beta-globin gene (human adult pattern of expression) but do express transcripts of epsilon- and gamma-globin genes (human embryonic and fetal pattern) at very high levels when exposed to a number of inducing agents. Results from this and other laboratories suggest that the control of this pattern of expression is mediated by the presence and/or absence of trans-acting factors which exert their action on sequences corresponding to the promoters of these genes. Sequence specific DNA binding proteins acting on cis-regulatory elements have been hypothesized to be key elements in eukaryotic gene transcription, and even though considerable progress has been made in their isolation, DNA binding proteins with affinity for the human globin gene promoters have not yet been identified. We have defined several positive and negative regulatory regions 5' to the epsilon-globin gene promoter, and detected binding of proteins to these regions.

The methodology used included DNase footprinting and the gel retardation assay. One of the negative regions defined binds to a factor present in high concentrations in non-erythroid cells. Using in vitro mutagenesis, inhibition of binding of a protein to this region causes a 10 fold activation of the epsilon-globin gene promoter in a CAT-assay. This technique has also allowed us to further define several positive and negative regulatory sequences 5' to the protein coding region of this gene.



NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25021-13 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Sickle Cell Anemia: The Intracellular Polymerization of Hemoglobin S

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

PI: Constance Tom Noguchi Research Physicist LCB, NIDDK

Others: Griffin P. Rodgers Senior Staff Fellow LCB, NIDDK
 Barbara Torain Biological Lab Technician LCB, NIDDK
 Alan N. Schechter Chief LCB, NIDDK

COOPERATING UNITS (if any) Hopital Henri-Mondor, Creteil, France (Dr. Y. Beuzard);
 LCDB, NIDDK (J. Blanchette-Mackie); University of Birmingham, U.K. (Dr. J.
 Stuart); Johns Hopkins University, Baltimore (Drs. G. Dover and S. Charache); MRC
 Unit, Kingston, Jamaica (Dr. G. Serjeant); MRC Lab of Mol Bio, Cambridge, England;
 Hopital de Bicetre, Paris, France (Dr. C. Poyart).

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Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

0.6

PROFESSIONAL:

0.3

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

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

The extent of intracellular polymerization of hemoglobin S is primarily determined by oxygen saturation, hemoglobin concentration and hemoglobin composition. We have examined the filterability of subpopulations of sickle erythrocytes to determine the possible extent of sickle hemoglobin polymer formation at arterial oxygen saturation which might adversely affect cell deformability. Progressive reduction of oxygen tension within the arterial range caused a sudden loss of filterability of sickle erythrocytes through 5 micron diameter pores at a critical pO₂, particularly in the dense cell fraction. This loss of filterability was reversible upon reoxygenation and occurred at a higher pO₂ than did morphological sickling. Impairment of erythrocyte filterability at high oxygen saturation suggests that small changes in oxygen saturation within the arterial circulation cause rheological impairment of sickle cells.

It has been appreciated that fetal hemoglobin has a specific "sparing" effect in inhibiting polymerization of sickle hemoglobin, however, the exact amounts of fetal hemoglobin necessary to ameliorate the various manifestations of the sickle cell syndromes have been uncertain. Epidemiological analyses of sickle cell disease severity and studies of the biophysics of intracellular polymerization were used to estimate potential clinical benefit of various levels of fetal hemoglobin, of increases in hemoglobin S solubility and of decreases in intracellular hemoglobin concentration for use as guideposts for therapeutic goals.

Erythrocytes containing hemoglobin Setif can undergo pseudosickling in the laboratory when incubated under select buffer conditions. Aggregation of hemoglobin lysate from these erythrocytes was detected when incubated in phosphate buffered saline at either 290 mOsm or 459 mOsm. The solubility of the lysate increased at 4 degrees C and the tendency for sickling decreased. Detailed studies of hemoglobin Setif aggregation may suggest alternate strategies of the inhibition of sickle hemoglobin aggregation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25025-12 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Specificity and Complement Binding Effect of Antigen-Antibody Interaction

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

PI: Hiroshi Taniuchi Chief, Section on Protein Chemistry and Conformation LCB, NIDDK

Others: Antonello Punturieri Visiting Fellow LCB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Protein Chemistry and Conformation

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

1.5

PROFESSIONAL:

1.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

It is well known that single amino acid substitution of antigenic determinants dramatically decreases the affinity to antibodies. This phenomenon is strikingly similar to our previous observations in amino acid substitution of the three-fragment complex of horse cytochrome c. As described in another report, we hypothesize that four closed loops consisting of contacting groups mediate delocalized interaction to generate extra energy to stabilize cytochrome c and that substitution of an amino acid which is a part of this closed loop would disrupt delocalized interaction. To investigate whether a specifically recognized amino acid is a part of a hypothetical closed loop formed across or within the interface between an antigen and an antibody we have isolated 7 monoclonal antibodies to yeast holo- or apo-iso-1-cytochrome c as described in the previous years. In the present studies, we have quantitatively determined the affinities of these monoclonals with respect to yeast holo- and apo-iso-1-cytochrome c, a panel of evolutionarily related cytochromes c, apocytochromes c, and homologous and hybrid fragment complexes. The results taken together with the previous data have permitted us to assign specifically recognized amino acids as follows. IgG monoclonals: 4-74-6, Leu 63 (yeast numbering) and/or Asn 67 and/or Asn 68; 4-126-6, Glu 93; 4-145-10, Thr 74; 2-96-12, Asp 65; 2-34-19, Lys 59; and 10-28-86, trimethyl-Lys 77. IgM monoclonal 39-14, Pro 30 and His 31. With the exception of mAbs 4-14-10 and 39-14 these monoclonals are of high affinity. A calculation with mAb 4-126-6 in which replacement of Glu 93 by alanine results in a decrease in affinity by a factor of 10,000 appears to show that the sum or conventional interactions such as electrostatics (or hydrogen bond), hydrophobic interaction and van der Waals interaction does not totally account for the decrease in affinity. Thus, we suggest that some new, extra interatomic interaction sensitive to differences in configurations of atomic groups may be involved in antigen recognition as predicted by the closed interaction loop hypothesis.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25028-10 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

The Development of Non-Invasive Methods to Assess Sickle Cell Patients

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

PI:	Griffin P. Rodgers	Senior Staff Fellow	LCB, NIDDK
Others:	Constance T. Noguchi	Senior Investigator	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

Clinical Hematology Branch, NHLBI (A.W. Nienhuis); Clinical Branch, NEI (M. Roy); Transfusion Medicine, CC (H. Klein); BEIB (Eli Walker); Biometry Branch, NEI (M. Podgor); MRC Laboratory, Kingston, Jamaica (G. Serjeant).

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SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

The molecular and cellular pathophysiology of the sickle cell syndromes are now appreciated with a great deal of precision. On the other hand, our understanding of the relationship of these subcellular events to the variable clinical expression of sickle cell disease remains largely speculative. A major effort of our research group has been to develop quantitative ways to clarify disease pathogenesis, as well as to assess severity and progression. Using calibrated phthalate ester separation method, which we previously described, we have now defined at least three cellular processes contributing to the extensive red cell heterogeneity that is commonly observed in the sickle cell syndromes. Ophthalmological studies of the patients show highly significant correlations between the extent of erythrocyte heterogeneity with conjunctival and retinal vessel pathology. As predicted by biophysical studies of polymer formation, we find that treatment of steady state sickle cell patients with selective arteriolar vasodilators results in a significant resolution of both acute conjunctival and retinal abnormalities, as well as an improvement in color vision performance. These beneficial effects occurred in the absence of a direct drug-induced inhibition of polymer formation, and therefore suggests that inappropriate vasospasm or frank vasoconstriction, perhaps in response to the altered rheology of red cell containing polymerized sickle hemoglobin is a significant contributing factor to the pathogenesis of sickle cell disease. This conclusion is also supported by our recent observation that "relative" hypertension is a significant risk factor for the occurrence of stroke in sickle cell patients. Using the technique of laser-Doppler velocimetry, we have found that forearm cutaneous microcirculatory flow undergoes a unique characteristic periodic pattern, which may become more "normalized" depending upon the fraction of non-S hemoglobins and during crisis. We hope that these cellular and physiological measurements will allow us to understand better the extreme spectrum of disease manifestations.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25038-08 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Effects of HTLV-I Tat-I Product on Globin Gene Expression

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

PI: Henry B. Fox Staff Fellow LCB, NIDDK

Others: Alan N. Schechter Chief LCB, NIDDK

COOPERATING UNITS (if any)

Metabolism Branch, NCI (Drs. T. Waldmann); LTCB, NCI (Drs. H. Streicher and R. Gallo)

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Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

0.8

PROFESSIONAL

0.8

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The control of human globin gene expression in erythroid cells involves trans-factors (substances active at distant locations in the genome), which have yet to be identified or clearly described. One experimental approach to their identification is to study the effects on globin gene expression of well-described trans-factors from tumor viruses. We have shown that the HTLV-I trans-factor tat-I stimulates both beta- and epsilon-promoters fused to a CAT gene, resulting in roughly 20-fold increase in CAT enzyme activity. In the case of beta-globin, only 185 bp of 5' flanking sequence is required for this effect. Recently, we have shown that stimulation of the epsilon-globin promoter requires at least 114 but not more than 177 bp of 5' flanking sequence. Computer analysis reveals 2 elements in this region homologous to the tat-I response element of HTLV-I; their functional significance is being investigated.

Further studies will involve characterization of the tat-I induced trans-activation of globin promoters. In progress are studies of the cis elements involved in tat-I responsiveness. These include fine structure genetic mapping by site directed mutagenesis of suspected response elements. Identification of these cis elements may lead to identification of DNA binding proteins involved in trans-activation of globin genes by tat-I. Our ultimate objective is to identify such cellular proteins that interact with tat-I to trans-activate globin genes. Study of such proteins may clarify the developmental regulation of globin gene expression in human erythroid cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Regulation of Globin Gene Expression by 5' Silencer DNA Sequences

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

PI:	Patricia Berg	Senior Staff Fellow	LCB, NIDDK
Others:	Ruo-Lan Qian	Visiting Fellow	LCB, NIDDK
	Shi-Xian Cao	Visiting Fellow	LCB, NIDDK
	Donna Williams	Staff Fellow	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

Laboratory of Molecular Hematology, NHLBI (Dr. R. Cohen)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

1.7

PROFESSIONAL:

1.7

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Although a number of human genetic diseases have been defined as mutations in or near the human beta-globin gene locus, regulation of gene expression of the human beta-globin gene is not well understood. To study this regulation, we are using a mutant human erythroleukemia cell line, K562. These cells can synthesize embryonic and fetal globins but not adult beta-globin, although they contain a structurally normal beta-globin gene which can be induced to express in transient heterokaryons. Thus, the molecular defect in K562 cells is most likely due to differences in trans-acting factors between K562 cells and normal erythroid cells such as continuous synthesis of a repressor, lack of synthesis of an activator molecule, or both. If there is a negative regulatory factor in K562 cells, deletion of its DNA binding site might then allow expression. On the other hand, deletion of DNA containing a binding site for a positive acting factor should be seen as decreased expression if the gene were active.

In an attempt to understand regulation of expression of the human beta-globin gene we are studying its 5' DNA sequences. Our deletion analysis of this DNA suggest there are at least three regulatory regions 5' to the beta-globin gene, two negative control regions (NCR) and one positive control region (PCR). Only the PCR appeared to be specific for K562 cells when these deletions were studied in a Chinese hamster and a mouse erythroleukemia cell line. Specific proteins which bind to NCR1 and NCR2 have been detected in nuclear extracts of uninduced and induced K562 cells and the DNA sequences to which they bind have been identified. There is a common protein which binds to both NCR1 and NCR2. There is also a protein binding to the region between them and to the positive control region. The significance of these binding proteins is under investigation.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25047-04 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Hydration Forces and Applications of the Osmotic Stress Technique

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

PI: Donald C. Rau Expert LCB, NIDDK

Others: Rudi Podgornik Visiting Fellow LCB, NIDDK

COOPERATING UNITS (if any)

Laboratory of Physical Sciences, DCRT and Laboratory of Biochemistry and Metabolism, NIDDK (Dr. W. Adrian Parsegian).

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Forces and Assembly

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25049-04 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Identification of a Silencer Element in the Human Epsilon-Globin Gene

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

PI:	Shi Xian Cao	Visiting Associate	LCB, MIDDK
Others:	Pablo Gutman	Visiting Fellow	LCB, MIDDK
	Harish Dave	Visiting Fellow	LCB, MIDDK
	Alan N. Schechter	Chief	LCB, MIDDK

COOPERATING UNITS (if any)

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Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

MIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

1.1

PROFESSIONAL:

1.1

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

DNA elements which negatively regulate transcription have been identified in many genes. One kind of these DNA elements which inhibits transcription in an position - and orientation - independent manner has been termed silencer. Silencers have been found in the rat insulin I gene, c-myc gene, mouse beta major globin gene, chicken lysozyme gene and in yeast, and appear to play an important role in the control of gene activity. We have studied the upstream control region of the human epsilon-globin gene. By deletion analysis, we found that a number of upstream regions have regulatory functions. Particularly, two negative regulatory regions located between -177 bp and -392 bp, and -535 bp and -1400 bp, and one positive regulatory region between -392 and -535 bp have been identified. As a first step to characterize these regulatory elements, we have concentrated on the region between -177 and -392 bp. This 220 bp fragment has been cloned in CAT expression plasmid containing 177 bp upstream sequences of the epsilon gene in both 5' and 3' positions and in both sense and antisense orientations. Upon introduction into the erythroleukemia K562 and HeLa cells, the plasmids containing the 220 bp DNA fragment showed significantly lower CAT activity compared with the plasmid without the fragment. This indicates that this fragment can act as a transcriptional silencer. To test whether this silencer functions in a heterologous system, we placed this fragment in the 5' side of the tk promoter. When transfected into HeLa cells, this silencer also inhibits tkCAT expression. Thus, the silencer not only functions on the epsilon-globin gene, it also functions on other promoters.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25050-04 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Structure and Physical Properties of DNA and DNA-Protein Complexes

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

PI: Donald C. Rau Expert LCB, NIDDK

COOPERATING UNITS (if any)

George Mason University, Fairfax, VA (Dr. H. Chen); LMB, NIDDK (J. Nickol); LCP, NIDDK (Drs. S.S. Wijemga and E. Charney); LCB, NHLBI (Drs. M.A.L. Atkinson and E.D. Korn).

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Laboratory of Chemical Biology

SECTION

Section on Molecular Forces and Assembly

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 BX 25951-14 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (20 characters or less. Title must fit on one line between the borders.)

Trans-Acting Factors Involved in Globin Gene Expression in K562 Cells

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

PI:	Helena Misrao	Staff Fellow	LCB, NIDDK
Others:	Donald Ray	Expert	LCB, NIDDK
	Paolo Guzman	Visiting Fellow	LCB, NIDDK
	Alan W. Schoedon	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

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Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOXES

<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

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

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25056-03 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Regulation of Human T Cell Receptor Delta and Alpha Gene Usage

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

PI: Jean-Pierre de Villartay Visiting Fellow LCB, NIDDK

Others: David Cohen Medical Officer LCB, NIDDK

COOPERATING UNITS (if any)

Washington University School of Medicine (Dr. S. Korsmeyer); Lab. of Tumor Cell Biology, NCI (Dr. E. Tschachler)

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Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The ability of T lymphocytes to recognize diverse ligands (antigens) resides in the T cell receptor (TCR), which is a heterodimer constructed from somatically rearranging variable (V) diversity (D) and joining (J) elements to account for its diversity, while each gene contains an invariant constant region. Most mature, effector T lymphocytes express the same class of TCR constant region, termed alpha/beta, but a smaller subclass of T cells appearing early in thymic ontogeny has been found to express a different heterodimer, termed gamma/delta. The function, the ligand, and the genetic regulation of this second receptor has been previously unknown although genetic clones for the gamma TCR existed. This project seeks to define and clone the delta TCR gene encoding the remaining chain of the second heterodimer, and to understand how genetic selection of the alpha/beta vs. gamma/delta TCR is accomplished.

During the last year, our laboratory and two other laboratories independently cloned the delta TCR gene utilizing the discovery that the delta TCR gene is tightly linked to the alpha TCR gene. These studies have established that the genetic order on human chromosome 14 is V-D-J delta-C delta-J alpha-C alpha, meaning that components of the alpha TCR flank the delta TCR on both sides. We have discovered an important regulatory rearrangement which deletes the delta TCR and which appears to be the initial step in alpha TCR gene usage. Consequently, a cell selects either gamma/delta or alpha/beta gene usage, so that these T cells derive from two different cell lineages. The regulatory rearrangement shares many common features with two immunoglobulin regulatory recombination events (class switching and kappa gene deletion). The study of the proteins involved in alpha versus delta TCR selection may lead to a better understanding of the regulation of gene rearrangement and may clarify the mechanisms of T cell maturation and selection within the thymus.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25057-03 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Function, Ligand, and Ontogeny of Expression of the Gamma/Delta T Cell Receptor

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

P.I.:	Amy B. Pullman	Guest Researcher	LCB, NIDDK
Others:	David I. Cohen	Medical Officer	LCB, NIDDK
	J.P. de Villartay	Visiting Fellow	LCB, NIDDK
	Lisa Jacobs	Biologist	LCB, NIDDK

COOPERATING UNITS (if any)

NIAD, NIH (Drs. J. Coligan and E. Shevach); NCI, NIH (Dr. Jeffrey Cossman); FDA (Dr. L. Matis).

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.5

OTHER:

.5

CHECK APPROPRIATE BOX(ES)

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

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

Effector T lymphocytes recognize antigens in association with a product of the major histocompatibility complex (MHC) through the T-cell receptor for antigen (TCR), a heterodimer composed of disulfide-linked alpha and beta chains. The appropriate pool of alpha/beta TCR-bearing cells is generated in the thymus upon positive and negative selection, a process called thymic education which eliminates TCRs with a high affinity for self MHC alone while expanding T cells bearing TCRs with affinity for self in association with foreign antigens. In addition to the alpha/beta TCR, a second TCR, termed gamma/delta, has been described on the surface of a small subset of immature T cells and thymocytes. This project aims to delineate the unknown function and the ligand(s) of the gamma/delta TCR. Because of its expression in immature T cells and thymocytes, it has been proposed that the gamma/delta TCR may play a role during the early events of thymic T cell development, including thymic education.

In order to dissect this project at the molecular level, we have first sought to define the complexity of the variable (V), diversity (D), and joining (J) element usage for the newly-described human delta TCR gene, because these elements combine to form the ligand-binding part of the receptor. We find that principally three V genes, 2 D genes, and only one J gene are used in the human contributing to all of the complexity of the gene. Genomic clones are being derived for each of these V genes, as well as for the D and J regions, which will be used to study the ontogeny of the gene's expression in human thymus. In addition, it has recently been possible to develop antigen-specific murine gamma/delta cell lines directed alternatively against class I MHC or class II MHC determinants. Genomic libraries generated from the DNA of these lines are used to isolate their antigen-specific gamma/delta genes as a first step toward developing transgenic mice bearing these genes. These transgenic mice will be an important in vivo model to study the function of the gamma/delta TCR.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25058-03 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Laboratory and Clinical Models for the Study of Globin Gene Expression

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

PI:	Griffin P. Rodgers	Senior Staff Fellow	LCB, NIDDK
Others:	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Nadera Ahmed	Guest Researcher	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

Lab. Mol. Genetics, NICHD (Dr. H. Westphal); MRC Unit, Univ. of West Indies, Kingston, Jamaica (Dr. G. Serjeant); Jackson Labs, Bar Harbor, ME (Dr. J. Barker)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

0.4

PROFESSIONAL:

0.1

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

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

We are investigating the molecular mechanisms which control the individual and total concentrations of hemoglobins in human erythrocytes. In addition, we are studying the effects of functional alpha globin gene number, fetal hemoglobin (HbF) levels and the extent of red cell heterogeneity on the various manifestations of sickle cell disease and its genetic variants. The levels of each of the normal hemoglobins (A, A2, F) are determined by controls at the level of transcription and/or translation of the globin genes, as well as by factors that regulate protein degradation. The study of the control of hemoglobin levels has direct relevance to various hemoglobinopathies, especially thalassemia and sickle cell disease. For our experimental system, we are using the K562 human leukemic cell line, as well as peripheral blood from individuals with sickle cell disease. We are studying the effects of short-term and long-term exposure of these cells to 5-azacytidine and hemin on their phenotype and the factors that control globin gene transcription. Adult beta-mRNA expression remains undetectable, yet we have found a constitutive level of another adult type hemoglobin, delta-mRNA, whose expression is inducible both with hemin and 5-azacytidine. Because of the close sequence homologies between the delta- and beta-globin genes, experiments are underway to examine whether changes in the delta-promoter sequence may alter important protein binding sites and thereby result in the low levels of delta-globin gene expression. Concurrently, we are also attempting to develop a sickle cell mouse model by the introduction of a cloned human sickle globin gene into the mouse germ line by the microinjection of DNA into the pronuclei of fertilized eggs. The establishment of such a model would allow for basic and fundamental questions to be asked about the molecular, cellular and physiologic aspects of the disease, as well as provide an in vivo system to monitor the effects of potential therapy.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25059-03 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Trans-activating Factors and Globin Gene Expression: A Direct Approach

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

PI:	Harish Dave	Visiting Fellow	LCB, NIDDK
Others:	Shi-Xian Cao	Visiting Associate	LCB, NIDDK
	Pablo Gutman	Visiting Fellow	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

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Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Humans undergo two developmental switches in their hemoglobin phenotype. The embryonic to fetal switch early in gestation and the fetal to adult switch around the time of birth. The K562 human leukemia cell line expresses all globin genes other than the adult beta-globin. Previous work from this laboratory has shown that the K562 beta-globin gene functions normally in a heterologous expression system. Elucidation of the mechanism of failure of beta-globin gene expression in K562 cells may provide an insight into globin gene expression and switching in normal erythroid cells.

A stable transformant system has been developed to permit the localization of sequences conferring tissue specificity to the upstream region of globin genes. It will also enable the in vivo titration of putative regulatory factor(s), thereby rigorously demonstrating the functional significance of certain sequences.

The direct isolation of trans-activating gene(s) will be attempted using the strategy that led to the isolation of several oncogenes. Hybrid beta-neo plasmids, which are expressed at a low level in K562 cells, will be co-transfected with another selectable marker (the MDR gene). Selection will be for the latter, followed by the identification of clones containing non-expressing beta-neo plasmid. K562 and MEL cell genomic DNA will be transfected into these cells and activation of beta-neo sought. A fractionation and/or "rescue" strategy will be employed to isolate the gene(s) of interest.

Known trans-acting factors such as TAT-1 of HTLV-1, the T antigen of SV40 virus and the Eta gene product of adenovirus will be studied in stable transformants.

A new cell line with a predominantly fetal phenotype has been characterized. Since no suitable human cell lines expressing beta globin are available, we will attempt to establish such lines from human bone marrow cells using oncogenes and/or origin deficient SV40 virus DNA.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25060-03 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

In Vitro Transcription of Human Globin Genes With K562 Nuclear Extracts

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

PI: Yuko Wada Visiting Fellow LCB, NIDDK

Others: Constance T. Noguchi Research Physicist LCB, NIDDK

COOPERATING UNITS (if any)

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SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

1.2

PROFESSIONAL

1.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

To characterize and isolate the trans-acting protein factors specific human globin gene expression, we have prepared nuclear extracts from hemin-induced and uninduced K562 cells for use in a soluble cell-free in vitro system (a run-off assay).

In order to examine the 5' promoter region which can affect transcriptional activity, the epsilon-globin gene promoter was truncated at 10 different sites by restriction enzymes. The variation in transcriptional activity of the epsilon-globin gene was observed depending on deleted region of promoter, suggesting the possible regions to which nuclear proteins involved in transcriptional process may bind.

K562 nuclear extracts can not initiate transcription from beta-globin gene. However, when K562 nuclear extracts are supplemented with HeLa whole cell extracts, beta-globin gene transcription is observed in vitro.

To identify the trans-acting proteins necessary for specific globin gene expression, the chromatographic fractionation of crude nuclear extracts of K562 cells are now in progress. The structural requirements for active gene transcription are being assessed by examining the binding characteristics of nuclear protein extracts from K562 cells to regions of the epsilon promoter and 2 kilobase 5'-flanking region, using gel-retardation electrophoresis, DNA-footprinting and ion exchange chromatography. To examine the functional requirements for transcription, a globin-hybrid gene system has been designed to facilitate the analysis, separation and recovery of viable cells in which the epsilon-globin gene is actively transcribed.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25061-03 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Isolation of Embryonic Globin Transcriptional Factors by Subtractive cDNA Cloning

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

PI: Yongji Wu Visiting Associate LCB, NIDDK

Others: Constance T. Noguchi Research Physicist LCB, NIDDK

COOPERATING UNITS (if any)

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Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

The specific transcription of human globin genes may involve a complex interaction of a variety of factors including trans-acting factors, none of which has thus far been completely characterized. The K562 human erythroleukemia cell line can serve as a model for the study of globin gene expression. The goal of the present study is trying to clone and characterize such factors.

The current study assumes that induced K562 cells contain transcriptional factors specific for embryonic and fetal globin genes, which are absent or present only at very low levels in uninduced K562 cells. For isolation of cDNA clones encoding the trans-acting factors, a cDNA library from mRNA of induced K562 cells, consisting of 450,000 independent recombinants, was constructed and 150,000 recombinants has been differentially screened. Seventy-five cDNA clones were found to hybridize only with cDNA probes from induced K562 cellular RNA and further examined by hybridization with Northern blot of induced and uninduced K562 cellular RNA. Forty-five cDNA clones have shown full length complements to the corresponding RNA. To determine whether some of the 45 cDNA encode trans-acting factors required to activate epsilon- or gamma-globin gene promoter, those cDNA have been inserted into Okayama-Berg expression vector and co-transfected into HeLa cells with another expression vector which contains epsilon promoter or gamma promoter and CAT gene or human growth hormone gene. One cDNA (No #17) has shown to be able to increase CAT gene expression about 2.5 times. This cDNA has been sequenced and is 522 nucleotides in length and contains an open reading from of 282 nucleotides. A search of the NBRF protein database revealed that this protein is not homologous to any known protein sequences. It is possible that the #17 cDNA encodes a protein or a part of a protein unknown before and involved in activation of epsilon-globin gene promoter. We are now continuing to examine the remaining cDNA clones using the same strategy.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25062-03 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Factors Affecting Mouse Beta-Globin Gene Expression

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

PI:	Donna M. Williams	Staff Fellow	LCB, NIDDK
Others:	Patricia Berg-Lovett	Senior Staff Fellow	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

Laboratory of Molecular Hematology, NHLBI (Drs. D. Kuebbing and W.F. Anderson).

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Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25063-02 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Effect of Hydroxyurea on Fetal Hemoglobin Synthesis in Sickle Cell Patients

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

P.I.	Griffin P. Rodgers	Senior Staff Fellow	LCB, NIDDK
Others:	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

CHB, NHLBI (A.W. Nienhuis); CB, NEI (Dr. M. Roy); BEIB (Mr. E. Walker); Depts. of Medicine, Pediatrics & Pathology, Johns Hopkins University, Baltimore, Md. (Drs. G. Dover and S. Charache); MRC Unit West Indies, Kingston, Jamaica (Dr. G. Serjeant).

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Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

0.4

PROFESSIONAL:

0.4

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

We have initiated a project to broaden the available fund of knowledge related to the effects of hydroxyurea (HU) on fetal hemoglobin synthesis in patients with sickle cell anemia by studying the acute and chronic responses associated with its administration to such individuals. These studies should provide insight into the pharmacokinetics of HU, optimal dosage regimens, and predictive factors associated with the F-reticulocyte response. Preliminary results on 8 patients treated continuously for a period of 3 months disclosed several interesting points. First, despite close monitoring and the achievement of adequate serum drug levels, some patients (3 of 8, in our initial series) will not respond to hydroxyurea. Second, other patients, who cannot be distinguished by current biochemical or molecular analysis, will obtain significant elevations of HbF, especially after long periods of treatment. This lag in response suggests that mechanisms in addition to acute cyto-reduction are involved in the effect of hydroxyurea on HbF production. Finally, the attainment of HbF levels in excess of 20-25%, in as pancellular a distribution as possible, may be necessary before objective indices of an improvement in microvascular pathophysiology are unambiguously stabilized or reversed. Should a significant sustained F-cell response be observed in select patients while on HU, it may be possible to increase further the magnitude of the response by simultaneously administering short courses of cloned human erythropoietin or cloned granulocyte-macrophage colony stimulating factor. In this fashion, one may approach fetal hemoglobin levels consonant with those observed in the benign HbS-HPFH phenotypes.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25064-02 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cytogenetic Investigations of Patients with Genetically Determined Diseases

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

PI: Beverly J. White Director, Cytogenetics Unit LCB, NIDDK

Others: Mary Graham Medical Technologist OD, CC
Zaven Kalayjian Medical Aid OD, CC

COOPERATING UNITS (if any)

Interinst. Med. Genetics Program, CC, (J. Mulvihill, D. Parry, J. Green); Genetics Dept., Children's Hospital Natl. Med. Ctr., Washington, D.C. (K. Rosenbaum); LN, NIA (M. Schapiro, S. Rapoport); CNG, NIMH (L. DeLisi, E. Gershon).

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Molecular Forces and Assembly (Cytogenetics Unit)

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

3.0

PROFESSIONAL:

2.7

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

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

In cooperation with the medical genetics program, cytogenetic methods were used to investigate over 200 patients participating in clinical protocols of the various institutes. Studies of Alzheimer's disease and adult Down syndrome patients were completed. The only constitutional variation correlated with dementia was typical trisomy 21. Secondary sex chromosomal aberrations of some experimental and control subjects were correlated with age but not disease. Data from cytological analysis of ribosomal DNA gene activity of the nucleolus-organizing regions (NORs) is being analyzed. A study of the NORs in Down syndrome parents and controls was concluded; NOR variants and duplication (dNOR) were not associated with trisomy 21. NOR activity was not significantly different in control and experimental groups and was not influenced by age and sex. Risk of having a child with Down syndrome could not be determined from NOR analysis.

Data from a collaborative study of the fragile X chromosome in 48 schizophrenic males and controls were reported. All affected subjects were fragile X negative; one individual with 47,XXX was found among controls. The fra(X) mutation at Xq27 may not be a major genetic factor in typical schizophrenia; the relationship of schizophrenia to mental disorders observed in some fra(X) carriers remains unexplained.

Our studies of recognized syndromes with high-resolution analysis confirmed the association of mental retardation, aniridia, and genitourinary anomalies with deletion of 11p13. No abnormalities were found in familial eosinophilia, familial pheochromocytoma, multiple endocrine neoplasia I, fibrodysplasia ossificans progressiva, McCune-Albright syndrome, Cockayne syndrome with xeroderma pigmentosum, and Beckwith-Wiedemann syndrome.

In situ hybridization experiments with high-resolution chromosome preparations and previously localized probes are still in progress, and collaborative work with unmapped DNA sequences is planned.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25065-02 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Transcriptional Control of Globin Genes in Human Erythroleukemia K562 Cells

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

PI:	Constance Tom Noguchi	Research Physicist	LCB, NIDDK
Others:	Griffin Rodgers	Guest Researcher	LCB, NIDDK
		Robert Wood Johnson Fellow	
	Nadera Ahmed	Guest Researcher	LCB, NIDDK
	Barbara Torain	Biological Lab Technician	LCB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been combined with Z01 DK 25060-03 LCB.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25066-02 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

AIDS: Transcriptional Regulation by TAT-Protein and LTR of HIV In Vitro

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

PI:	Jiangang Yuan	Visiting Fellow	LCB, NIDDK
Others:	Constance Noguchi	Research Physicist	LCB, NIDDK
	Henry Fox	Medical Staff Fellow	LCB, NIDDK
	Alan Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

Kabigen, Stockholm, Sweden (Professor M. Hartmanis)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS

1.4

PROFESSIONAL:

1.4

OTHER

0

CHECK APPROPRIATE BOX(ES)

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

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

The HIV-retrovirus is the etiologic agent for AIDS. The high level of viral expression is attributed, at least in part, to the virus-encoded transactivator protein TAT, which has the ability to autoregulate the expression of HIV by promoting the transcriptional activity of the HIV LTR promoter. The molecular cloning of HIV has provided the isolation of TAT coding DNA and LTR promoter sequence from other HIV sequences and has facilitated studies of the TAT protein on the LTR promoter independent of other retroviral proteins.

In the past year, we used an in vitro cell-free transcriptional system to study the transacting activity of the TAT protein on the HIV LTR promoter and other virus and cellular gene promoter. The effect of the TAT protein on the expression of genes directed by different promoter were compared by using nuclear extract of HeLa/t2 cell, which constitutively expresses TAT protein. We found that the HeLa/t2 nuclear extract markedly enhanced the transcription from HIV LTR promoter, but did not show any enhancement of transcription from beta-globin, epsilon-globin and adenovirus MLP promoters. Furthermore, when various amounts of HeLa/t2 nuclear extract were added to the transcriptional reaction mixture, the amount of the specific RNA product from HIV LTR was increased proportionally. The results, therefore, suggest that the TAT protein may be a specific trans-activating factor for the HIV LTR promoter. Further studies with purified TAT protein from prokaryotic cell, using cloned TAT gene in expression vectors, is being done to be able to provide directly evidence for the transacting activity of TAT protein and to develop assays for the study of potential inhibitors of the protein. Large scale production, isolation and purification of the tat protein from prokaryotic cells using cloned tat DNA has yielded a small amount of tat-protein (a few micrograms). However, the method of production has resulted in extensive degradation of the protein product. This work could lead to a new approach to the prevention or treatment of AIDS.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25067-01 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Genetic Control and Mechanism of Action of Erythropoietin

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

PI: Henry B. Fox Staff Fellow LCB, NIDDK

Others: Inyoung Ko Guest Researcher LCB, NIDDK

COOPERATING UNITS (if any)

AFRRI, Defense Nuclear Agency, Bethesda, Maryland (Dr. W.D. Hankins and Ms. K. Chin)

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Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.2

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The recently described mouse cell lines HCD-9 and SC-10 are derived from the Friend Murine Leukemia Virus. These cells absolutely require added erythropoietin for growth in culture. These are the first such cell lines described. We have further characterized these cell lines and demonstrated erythroid differentiation in response to hemin: morphologic maturation, benzidine stain positivity, and increased globin RNA content. Further studies are focusing on the erythropoietin receptor in these cells.

We have also studied the mouse cell lines IW-32 and TP-3. These cells abnormally secrete erythropoietin. We have cloned the erythropoietin gene and flanking DNA regions from each cell line. Thus far, IW-32 shows a rearrangement 5' to the gene by restriction mapping. We are sequencing both this gene and its normal counterpart. Future studies will assess the functional significance of this rearrangement. We will also look for mutations affecting control of the gene in TP-3.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25068-01 LCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Regulation of Globin Gene Expression by Upstream Positive Control DNA Sequences

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

PI:	Moshe Mittelman	Visiting Associate	LCB, NIDDK
Others:	Patricia Berg	Senior Staff Fellow	LCB, NIDDK
	Donna Williams	Staff Fellow	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS:

1.6

PROFESSIONAL:

1.6

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The regulation of expression of the human beta globin gene is still not well understood. Ongoing studies in our laboratory (Project #Z01 DK 25045-04 LCB) focused on the 643 base pairs (bp) upstream from the human beta globin gene. A series of deletion analyses, followed by transient expression assays and chloramphenicol acetyl transferase (CAT) assays revealed 3 upstream DNA regions of regulatory importance. Two regions (between -643 and -490, and between -338 and -233) were found to have a negative effect on gene expression, since their deletion increased the CAT activity, and they were defined as Negative Control Region I (NCR1) and NCR2 respectively. Deletion of a third region (between -233 and -185) was followed by a dramatic drop in CAT activity, suggesting a positive role of that segment, thus defined as Positive Control Region (PCR).

While the other control regions are currently being investigated, we are focusing on the PCR. The positive control effect was detected in a human erythroid cell line (K562) but not in a mouse erythroleukemia (MEL) or a Chinese Hamster (R1610) cell lines, suggesting a human erythroid tissue specific activity, in contrast with the NCRs. This specificity, as well as the proximity of the PCR to NCR2 raise the possibility that PCR may contain an enhancer sequence, namely a DNA sequence capable of increasing the promoter activity and hence the gene expression in a position and orientation independent manner. This speculation is based on reports describing enhancers as tissue specific and controlled by close negative control elements which repress the gene expression in other tissues, while the repression is removed in the specific tissue enabling the gene to be expressed (prealbumin liver cells, insulin in beta cells, etc.).

In an attempt to understand the PCR role, we are cloning it (100 bp) into two BamHI sites of the -185 deleted form of the fusion plasmid p-beta-GLCAT. Transfection of hemin induced K562 cells with the new DNA recombinants may answer the question of the PCR as an enhancer.

ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research in the Laboratory of Chemical Physics is primarily concerned with the investigation of problems in molecular and cellular biophysics. A variety of spectroscopic techniques are employed in these investigations, including nuclear and electron magnetic resonance, Raman and infrared spectroscopies, electric-field-induced linear dichroism, ultraviolet and visible microspectrophotometry, and time-resolved optical spectroscopy using nanosecond lasers. There is also a major effort in theoretical studies to complement the experimental work, including both analytic methods and the use of high speed computers in large scale calculations. The systems under study include nucleic acids, proteins, intact and model membranes, retinal photoreceptors, and various small prototypical biological molecules. Current research focusses on: the development of new methods in nmr; the structure of proteins in solution by two-dimensional nmr; the structure and dynamical behavior of nucleic acids and nucleoproteins; conformational, dynamical, and functional characteristics of model membrane systems; the dynamics of ligand binding and conformational changes in proteins; theoretical analysis of kinetics and dynamics in macromolecules; computer simulations of atomic motions in proteins; rheological properties of cell membranes; the molecular mechanism of excitation in photoreceptor cells and ionic processes in cell membranes; the gelation of hemoglobin S and its relation to the pathophysiology of sickle cell disease; the analysis of excited electronic states of polyenes in the vapor phase and in molecular beams; and the asymmetric synthesis and structure of metabolites; The following gives a brief summary of the major findings over the past year.

Bax and colleagues have developed a number of new methods for the determination of three-dimensional molecular structures in solution by nuclear magnetic resonance measurements. Bax and Marion have developed an improved version of the ^1H -detected heteronuclear chemical shift correlation technique to gain considerable improvement in both resolution and sensitivity. Bax's earlier version has been widely used for structure determination of organic molecules, while the improved method has been applied by Bax and Torchia to resonance assignments in proteins such as staphylococcal nuclease.

Torchia, Sparks, and Bax have developed new methods based on isotope substitution which permits the investigation of the full three dimensional structure of proteins of considerably higher molecular weights. The new methods involve labelling proteins with the stable isotopes - ^2H , ^{13}C , and ^{15}N - to simplify the spectra and permit the acquisition of very high quality NOE spectra from which interatomic distances are derived.

Marion and Bax have used 2D nuclear magnetic resonance techniques to study the antimicrobial peptide, magainin, discovered by Zasloff (NICHD). In aqueous solution the peptide possesses no well-defined structure, but readily adopts an alpha helical conformation upon addition of small amounts of trifluoroethanol. The solution of the complete structure, including side chain conformations, using computer modelling based on homo- and heteronuclear J couplings and a very large number of interresidue NOE's is in progress.

Clore, Gronenborn, and coworkers have been determining the complete three dimensional structure of small proteins in solution using a combination of distance restraints from nuclear magnetic resonance measurements and annealing of the structures using molecular dynamics. Driscoll, Clore, and Gronenborn have determined the structure of a 43-residue protein BDS from *sea anemone* using the new 600 MHz spectrometer. BDS is an interesting and important protein because it lowers blood pressure without being either neuro- or cardiotoxic, and exhibits antiviral activity against mouse hepatitis B virus. Because of the large number of distance and torsion angle constraints, the average root mean square difference between individual structures calculated from the molecular dynamics annealing was only 0.6 angstroms for the backbone atoms and 0.9 angstroms for all atoms. This is the most accurately determined structure by nuclear magnetic resonance for a protein.

Folkers, Clore and Gronenborn are investigating the three-dimensional structure of the protein hirudin and an active-site mutant by nuclear magnetic resonance. Two-dimensional nuclear magnetic resonance techniques have been used to make complete sequential assignments of the proton resonances, and 700 interproton distance restraints have been acquired. The data so far suggest that the single amino acid change in the active site (lys47 \rightarrow glu) produces small, but significant changes in the structure. This protein from the leech, which has 65 residues, is the most powerful known natural inhibitor of coagulation. It binds to thrombin, and inhibits the conversion of fibrinogen to fibrin.

Electric birefringence and dichroism are being used by Charney and coworkers to investigate the conformation of DNA and other biological polyelectrolytes in solution. Studies on hyaluronic acid have demonstrated that the theory previously developed by Charney and coworkers is consistent with the experimental results. Experiments on the A form of DNA show that the orientational decay is insensitive to ionic strength in a range where the B form of DNA is extremely sensitive. This may result from differences in conformational flexibility, and that idea is currently being explored with further experiments on random sequence fragments.

Levin and coworkers are using a variety of vibrational spectroscopic techniques to study the structure and dynamics of biological membranes and model systems. Raman spectroscopy has been employed by Levin and Vincent to investigate the weak interactions between zwitterionic lipids and extrinsic membrane proteins. In liposomes and multilayers of 1- α -dipalmitoylphosphatidylcholine reconstituted with very low concentrations of ferricytochrome c, the protein affects the disorder in the bilayer under conditions which show no influence of either pH or ionic strength. Furthermore, the gel to liquid crystalline phase transition occurs at a lower temperature.

Lewis, Kalasinsky, and Levin have developed a new technique for measuring Raman spectroscopy using Fourier-transform techniques and excitation with a near infrared laser. Near-infrared excitation eliminates interference from fluorescence, which is a major problem with most biological samples, while the use of fiber optics is shown to increase the sensitivity of the measurements by allowing much more precise optical alignments at much lower power densities of the incident laser. These new methods have many additional advantages over

conventional techniques and should make many more biological systems accessible to the Raman spectroscopic technique. One of the important applications by Levin and coworkers has been to examine the effects of derivatives of amphotericin B on membrane fluidity. This study suggests that the antiviral effect of these compounds on HIV infectivity may result from introducing significant structural defects in the virion envelope.

Transient spectroscopy using pulsed nanosecond lasers has been used by Hofrichter, Henry, Eaton and coworkers to study structural changes in proteins following optical excitation. The performance of the transient spectrometer has been considerably improved by Hofrichter and Henry by developing new software control of the vidicon detector and programs for data analysis. Murray, Hofrichter, Henry, and Eaton are carrying out a detailed transient spectroscopic study of ligand rebinding to hemoglobin following photodissociation of the carbon monoxide complex. Using the iron-cobalt hybrid molecule they have shown that the 60-fold reduction in the overall association rate of the alpha subunit in the T quaternary structure compared to the R quaternary results from a decreased binding rate at the heme. There is no major effect of the quaternary structure on the rate of entry of the ligand into the protein or the rate of exit of the ligand from the protein. In order to carry out a similar study for the beta subunit, measurements were carried out with a hybrid molecule in which the iron in the alpha subunits was replaced with the unreactive nickel(II) ion. These studies show that the decreased binding rate for the beta subunit in the T state also results from slower binding at the heme. The scaling of the bimolecular rates and the geminate processes for both the alpha and beta subunits, which occur on a time scale of about 50 nanoseconds, suggests that functionally significant conformational changes occur on a subnanosecond time scale. Eaton in a collaboration with Hochstrasser at the University of Pennsylvania have employed picosecond lasers to examine the structural changes in hemoglobin between 30 picoseconds and 10 nanoseconds. The lack of any spectral changes of the heme in this time regime following photodissociation of carbon monoxide suggests that the functionally significant tertiary conformational changes occur on a time scale of less than 30 picoseconds.

Hofrichter and Lozier have used the transient spectrometer to carry out an investigation of the photocycle of bacteriorhodopsin, the photoactive proton pump from the purple membrane of halobacterium Halobium. By measuring very precise absorption spectra following optical excitation, a model has been proposed in which the photoinduced cis-trans isomerization produces at least two distinct molecular species with the retinal chromophore in the all-trans configuration.

Eaton and Hofrichter have continued their investigation of the gelation of hemoglobin S and its relation to the pathophysiology of sickle cell disease. The results of a study on the effect of carbon monoxide saturation on the delay time of sickle cell hemoglobin gelation has been analysed by Henry. The data show that the effect of saturation on the delay time reflects the change in solubility with saturation, with a slow increase in delay time with saturation at low saturations, and a dramatic increase in the delay time at the higher, physiologically-significant saturations.

Szabo has carried out theoretical investigations on the functional significance of dynamical processes in several different biological systems.

In collaboration with Pastor (FDA), Venable (FDA), and Karplus (Harvard University) he has carried out a Brownian dynamics simulation of a hydrocarbon chain in a membrane bilayer. The results support a very fluid picture of the interior of the bilayer, much like a neat alkane in both the types and timescales of torsional reorientation, in sharp contrast to the widely-accepted crankshaft models of internal motions. In a separate study, Szabo has used the stochastic theory of chemical reactions and the theory of first passage to derive an analytical expression for the distribution of delay times when sickle hemoglobin polymerizes in small volumes, such as in red cells. The theory permits the direct determination of the rate of homogeneous nucleation, and represents one of the very few examples where contact has been made between experiment and the stochastic theory of chemical reactions. Szabo has also collaborated with Kamp (LMB) to investigate the influence of conformational dynamics on electron transfer reactions. This study showed that the exact flux of electrons from the donor to the acceptor is identical to that calculated for a simple four-state kinetic scheme if the effective rate constants of this scheme are appropriately defined in terms of the mean first passage times for moving between various points of a four-state cycle.

Hagins and Yoshikami have used a combination of optical imaging, calorimetric, and electrophysiological techniques to investigate the mechanism of visual transduction. By comparing the magnitude and time course of heat production by vertebrate retinal photoreceptors in response to flashes of visible light Yoshikami, Tate, Ross (LMB), and Hagins have demonstrated significant discrepancies between observed heats and those predicted by the model in which cyclic GMP controls the dark current conductance. Another inconsistency with the cyclic GMP model is the finding that the initial heat pulse remains at its full size when the dark current is suppressed by calcium, suggesting that it acts directly on the dark current conductance and not through cyclic nucleotide metabolism. By investigating the pattern of heat production over the full physiological range of stimuli components corresponding to almost all of the postulated reactions of the cyclic GMP model have been identified and measured. From measurements of dye diffusion in rod outer segments using improved video microscopy Yoshikami and Spring (NHLBI) have shown that the intracellular membranous discs obstruct cytoplasmic diffusion. Additional observations place bounds on both the electrolyte content of the rods and the amount of cyclic nucleotide hydrolysis that must occur during phototransduction.

Kon and coworkers have continued their investigation of red cell deformability using electron spin resonance techniques. Kon and Fukushima have shown that the low deformability of resealed red cell ghosts can be considerably improved by treatment with Mg-ATP, chlorpromazine, or calcium ions during resealing. The novel finding of the deformability enhancing effect of calcium is assumed to be caused by electrostatic expansion of the inner leaflet of the membrane bilayer relative to the outer layer due to the extra positive charges introduced by the divalent calcium. Kon and Riesz (NCI) have shown hemolysis of red cells induced by ultrasound is the result of physical damage due to the shear stress of the sonication and is not caused by chemical reactions resulting from the free radicals generated during sonication. Measurements of membrane fluidity, permeability and deformability indicate that cells which survive hemolysis have virtually no alterations in structure due to sonication.

McDiarmid has continued an investigation of the electronic states of butadiene and other simple polyatomic molecules as models for more complex biological molecules. An investigation of the electronic spectrum of 2,3-dideuterobutadiene has resulted in a reinterpretation of the vibrational substructure of the $V \leftarrow N$ transition. Electronic states analogous to this were studied in cyclopentadiene, with the novel finding of an excited electronic state that is lower in energy than the observed state in long polyenes. Spectroscopic studies on acetone resolved a controversy regarding the energy ordering of several excited states of this molecule.

Ziffer and coworkers have carried out several new types of organic syntheses. An antibody combining site was shown to selectively promote the formation of an isomer by serving as a template. To achieve this effect antibodies were raised against an analogue of one of the photoproducts of trans methyl p-nitrocinnamate. Irradiation of a solution in the presence of antibodies resulted in preferential formation of the isomer to which the antibodies were raised. In a separate study a general method was developed for resolving polycyclic aromatic alcohols, metabolites of polycyclic aromatic hydrocarbons.

Scientists Emeritus Weiss in collaboration with Cook (University of Wisconsin) has continued to explore the "Weiss reaction" to synthesize a wide variety of cyclopentanoid structures (also called polyquinanes). These compounds include many natural products. They are also useful for the study of molecular properties such as unusual molecular conformations and the presence or absence of aromatic character.

Sharpless has continued his investigations of molecular conformation using molecular mechanics and molecular orbital calculations. In one study it was shown that for the AIDS drug AZT the barrier to rotation of the base is quite low (< 5 kcal/mole).

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Molecular dynamics and vibrational characteristics of membrane assemblies

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

P.I. : Ira W. Levin Research Chemist LCP-NIDDK

Others: E. Neil Lewis Visiting Associate LCP-NIDDK

Mark Devlin IRTA LCP-NIDDK

Victor F. Kalasinsky IPA LCP-NIDDK

COOPERATING UNITS (if any)

R. Adams, LCP-NIDDK; Clifford J. Steer, LBM-NIDDK; C. Haung, School of Medicine, Univ. of VA; James S. Vincent, Univ. of MD.

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS

4

PROFESSIONAL:

4

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Vibrational Raman and infrared spectroscopy are used to probe the dynamical, conformational, functional and thermodynamic properties of both model and intact membrane assemblies. Emphasis is placed on elucidating both lipid-lipid and lipid-protein interactions within the bilayer aggregate. For example, the association between ferricytochrome c and hydrated zwitterionic phospholipid bilayers comprised of dipalmitoylphosphatidylcholine (DPPC) were studied using Raman spectroscopic techniques. The protein-lipid reconstituted liposomes were examined under varying conditions of protein concentrations, pH and ionic strength; spectra were reported as a function of temperature. The two most important spectral scattering parameters used to monitor bilayer order/disorder characteristics were total, integrated band intensities and relative peak height intensities. These quantities, which reflected a variety of intramolecular and intermolecular processes, are invariant to changes in pH and ionic strength, but were sensitive to protein concentration. Ferricytochrome c and DPPC form non-stoichiometric complexes capable of altering bilayer packing properties and the temperature behavior of the liposomal assemblies. For the extrinsic ferricytochrome c protein, concentrations of $\sim 10^{-4}M$ to $<10^{-5}M$ result in bilayer penetrations which lead to significant membrane perturbations.

A new technique, Fourier-transform Raman spectroscopy using near infrared Nd:YAG laser excitation, was developed for determining Raman spectra of highly fluorescing biological samples. (Raman spectra of fluorescing systems cannot be obtained when conventional, visible laser techniques are used with dispersive systems.)

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Chemistry of natural compounds, and synthetic organic chemistry

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

P.I.: Ulrich Weiss Research Chemist (Scientist Emeritus) LCP-NIDDK

COOPERATING UNITS (if any)

Prof. James M. Cook, Department of Chemistry, University of Wisconsin-Milwaukee

LAB/BRANCH

Laboratory of Chemical Physics

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TOTAL MAN-YEARS:

1

PROFESSIONAL:

1

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

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

In continued cooperation with Prof. J.M. Cook and his coworkers at the University of Wisconsin-Milwaukee, the synthesis of di- and polycyclic ring systems composed of fused cyclopentane rings ("polyquinanes") has been developed further. The approach chosen is based on the ready stereospecific formation of derivatives of cis-bicyclo[3.3.0]octane-3,7-dione (1) from 1,2-dicarbonyl compounds and esters of 3-oxoglutaric acid (the "Weiss reaction").

Several more tetracyclic polyquinane ring systems have been synthesized by suitable further transformations of (1) or its closely related analogs. Thus, the first 1, 10-disubstituted derivatives of the long-known, triply unsaturated triquinane triquinacene (R.B. Woodward et al., 1964) have been made, including a novel compound, 2, where an additional six-membered ring is fused at these positions of triquinacene. Saturated representatives of several other systems have been prepared as intermediates in the planned synthesis of related compounds with the maximum possible number of double bonds; these latter molecules should be valuable for intended studies of the possible aromatic nature of such compounds.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-19005-14-111

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Asymmetric synthesis: structure, stereochemistry and NMR

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

P.I.:	Herman Ziffer	Research Chemist	LOP-NIDDK
Other:	Yulin Hu	Visiting Fellow	LOP-NIDDK
	Ayala Balan	Visiting Scientist	LOP-NIDDK

COOPERATING UNITS (if any)

Dr. J. V. Silvertown NHLBI, Dr. Sanford Markey NIMH, Dr. Mark Duncan NINCDS, Dr. Bernard S. Green, Hebrew University, Israel.

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1

PROFESSIONAL

2

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

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

Antibodies were raised against an analogue of one of the photo products (the head-to-head syn dimer) of trans methyl p-nitrocinnamate. When a solution of the monomer was irradiated in the presence of these antibodies, the product formed preferentially was the one against which antibodies had been raised. Photoisomerization of the monomer, the major photochemical reaction in the absence of antibodies, was greatly suppressed. Thus, the antibody combining site serves as a template for promoting selectively one reaction. The structure and absolute stereochemistry of (Z) (+)-2-bromocycloundec-2-enyl camphanate, a compound prepared as part of a study of the relationship between the chiroptical properties of the corresponding p-bromobenzoate esters and the configuration of conformation of medium-ring allylic esters, was determined in an X-ray crystallographic study. In the course of the same study, the structure and absolute stereochemistry of (E) (-)-2-cyclododecyl camphanate was also determined by X-ray crystallography.

As part of our work on asymmetric synthesis we developed a general method of resolving polycyclic aromatic alcohols, metabolites of polycyclic aromatic hydrocarbons. This method was used to prepare (+)-1-acenaphthenol, a substrate used to analyze several dehydrogenases. The absolute stereochemistry of the camphanate ester of the alcohol was elucidated by an X-ray crystallographic study.

Chiral samples of β -methylaminoalanine (BMAA) a reported neurotoxin and of an ^{15}N labeled sample were prepared utilizing an enzymic resolution. The metabolism of the reported neurotoxin and as well as its pharmacology are being investigated by Dr. Markey et al. in NIMH see Z01-MH-00279-05-LCS.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29006-18-LCP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

The structure and dynamics properties of macromolecules

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

P.I. : Elliot Charney Research Chemist LCP-NIDDK

Other: Sybren Wijmenga Visiting Fellow LCP-NIDDK

COOPERATING UNITS (if any)

H-H Chen, George Mason University, Fairfax, VA; Rodney Harrington, University of Nevada, Reno, Nevada; D.C. Rau, LCB-NIDDK; Sybren Wijmenga, University of Nijmegen, The Netherlands

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Laboratory of Chemical Physics

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Section on Spectroscopy and Structure

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

1.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Macromolecular structure, dynamics and polyelectrolyte properties of large biological polymers, in particular, polynucleotides and nucleic acids are being studied by electric-field induced dichroism and birefringence methods. Theoretical and computational methods supplement the experimental work.

The current research is a response to the fact that the knowledge of the structural effects of specific base-pair sequences on DNA translation and replication is still at a primitive stage. Only one or two biologically significant protein-DNA complexes from which such structural effects could be inferred have been crystallized and their structure determined. Using electro-optic birefringence and dichroism, it is now possible to quantitatively explore DNA structures in solution, albeit with less resolution than x-ray diffraction of crystals, but uninhibited by the problem of forming crystalline complexes. The two principal projects currently being pursued are the structural effects of the triplet sequence CAC/GTG and the flexibility of the A form of DNA.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29007-17-LCP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Structure and interaction of biomolecules

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

P.I. : Hideo Kon Research Chemist LCP-NIDDK

Others: Yasunori Fukushima Visiting Fellow LCP-NIDDK

COOPERATING UNITS (if any)

P. Reisz, NCI-ROB

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TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The project aims at applying electron paramagnetic resonance (EPR) to probing structure and function of biological systems, and attempts to develop a new mode of application. Results of collaborative effort in 1988 include investigation of the mechanism of hemolysis induced by ultrasound (50 KHz) sonication; hemolysis was shown to be the result of physical damage due to shear stress attending sonication and not by the chemical insult of free radicals generated during sonication. The membrane fluidity, permeability, and deformability measured on the remaining erythrocytes after sonication are identical to the control suggesting that the cells that survived hemolysis have virtually no alterations in the structure due to sonication. The gamma radiolysis (700 Gy), on the other hand, was shown to cause significant damage to the structure of the surviving cells most likely as the result of free radical attacks. In our own project, the flow EPR technique devised in this laboratory for assessing erythrocyte deformability in flow was applied to studying the change of flow characteristics of resealed ghosts when the variables in ghost preparation are changed to investigate the rheological characteristics of membrane system itself apart from the influence by the cell contents. The ghost deformability in flow was found to improve over that of the control ghost by the presence of Mg-ATP, chlorpromazine, or Ca^{2+} ions at the time of resealing, and by lysing the cell pseudo-isotonically in NH_4HCO_3 solution with less osmotic stress than in ordinary hypotonic lysis. The improved ghost deformability was explained by the mechanism of recovery of natural balance in the bilayer system (including the cytoskeletal network) by these agents. Such balance is assumed to be perturbed during hypotonic lysis e.g. by transfer of inner layer phospholipids to the outer layer.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29008-17-LCP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Electronic and molecular structural investigations

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

P.I. :	Ruth McDiarmid	Research Chemist	LCP-NIDDK
Others:	Abdol-Hakim Sheybani-Nezhad	Visiting Fellow	LCP-NIDDK
	Andrea Adams	Summer Student	LCP-NIDDK

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Leo Klasinc, Rudjer Boxkovic Institute, Yugoslavia

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Laboratory of Chemical Physics

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TOTAL MAN-YEARS:

PROFESSIONAL

OTHER:

1.25

1.25

CHECK APPROPRIATE BOX(ES)

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

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

The measurement and analysis of the spectrum of the V <- N transition of 2,3-dideuterobutadiene has lead to the reinterpretation of the vibrational substructure of this transition. The diffuse subbands observed were shown to arise from transitions to mixed normal modes of the excited state, not to transitions to a progression in the C=C stretching vibration of the molecule. The diffuseness of the transition was shown to be due to rapid nonradiative relaxation through or over a very small barrier in the C=C torsion to a almost degenerate electronic state. Through an investigation of the spectral emission of tetramethyl butadiene, the energy ordering of the two states in question was shown to be inverted in TMB from that observed in butadiene. The probably analogue of the acceptor state in butadiene was observed for the first time in any diene in the essentially cis isomer, cyclopentadiene.

A polarization-selected resonant multiphoton ionization spectroscopic investigation of acetone was conducted to resolve existing questions concerning the excited state energy ordering in that molecule.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Studies on sickle cell disease

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

P.I. : William A. Eaton Medical Officer LCP-NIDDK

Others: James Hofrichter Research Chemist LCP-NIDDK

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.75

PROFESSIONAL:

.75

OTHER:

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

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

A quantitative description of the role of gelation in the pathophysiology of sickle cell disease is being formulated to aid in the development of agents that can be used in the treatment of patients. A new laser photolysis technique has been developed to assess the quantitative significance of the delay time of hemoglobin S gelation to the pathophysiology. The saturation at which polymers first form in individual sickle erythrocytes upon deoxygenation is much lower than the saturation at which polymers disappear upon reoxygenation. The results indicate that at physiological saturations with oxygen, gelation takes place in the large majority of cells at equilibrium, but is prevented from occurring in vivo because the delay times are sufficiently long that most cells return to the lungs and are reoxygenated before polymerization has begun. These techniques are being extended to measure the delay time as a function of saturation on physiological times scales over a wide range of hemoglobin S concentrations and saturations. With these data it will be possible to provide a more accurate description of gelation in vivo. The measurement of the delay time on single cells in these experiments can also be used as a very sensitive method to assess the potential efficacy of agents that are potential drugs for the treatment of sickle cell disease. The measurement of the distribution of delay times at zero saturation will be automated to permit examination of a large number of agents, to compare intracellular gelation and clinical severity in patients, and to follow changes in intracellular gelation in patients on various therapeutic protocols.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29010-16-LCP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Conformation and electronic structure of biological molecules

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

P.I.: William A. Eaton Medical Officer LCP-NIDDK

Others: James Hofrichter Research Chemist LCP-NIDDK

Eric R. Henry Research Physicist LCP-NIDDK

Lionel P. Murray Staff Fellow LCP-NIDDK

COOPERATING UNITS (if any)

Takashi Yonetani and Masao-Ikeda-Saito, University of Pennsylvania of School of Medicine; Bernard Brooks, DCRT; Robin M. Hochstrasser, University of Pennsylvania; Maurizio Brunori and Massimo Coletta, University of Rome.

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

HIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

.75

PROFESSIONAL:

.75

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Time resolved optical spectroscopy with nanosecond lasers and molecular dynamics calculations have been employed to investigate ligand rebinding and conformational changes in hemoglobin subsequent to photodissociation of the carbon monoxide complex. In order to precisely measure the time course of the changes in the conformation of the deoxy photoproduct, which produce small spectral changes, as well as to determine the kinetics of ligand rebinding, an automated, sensitive nanosecond spectrometer has been developed to measure time-resolved spectra. The spectra have been analyzed using singular value decomposition to produce a set of orthonormal basis spectra and the time course of their amplitudes. With these techniques the kinetics of ligand rebinding and conformational changes have been studied with hemoglobins initially in the R and T quaternary structure. The R to T quaternary transition is observed for the completely unliganded R state molecule to occur at about 20 μ s, while both R and T state molecules show tertiary conformational relaxations at about 50 ns and 500 ns. The 50 ns relaxation is simultaneous with geminate rebinding, suggesting that it is caused by motion of the ligand out of the heme pocket. Using the simplest kinetic model, a comparison of the geminate kinetics for R and T state molecules indicate that the difference in the factor of about 50 in the overall rate of ligand binding to the R and T states can be explained by differences in binding rates to the heme from within the heme pocket. Changes in the barriers to motion of the

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

The physics and chemistry of photoreception

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

P.I. : William A. Hagins Medical Officer LCP-NIDDK

Others: S. Yoshikami Research Biologist LCP-NIDDK

F. M. Hagins Geust Worker LCP-NIDDK

M. C. Foster Research Physicist LCP-NIDDK

P. Ross Research Chemist LMB-NIDDK

K. Spring Research Med. Officer LKM-NHI

R. Tate Computer Systems Analyst CSL-DCRT

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Membrane Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

An investigation of the mechanism of phototransduction in vertebrate photoreceptor cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

The influence of molecular structure on chemical and biological properties

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

P.I. : Norman E. Sharpless Research Chemist LCP-NIDDK

Others: Ralph G. Adams Research Physicist LCP-NIDDK

William H. Jennings Research Physicist LCP-NIDDK

COOPERATING UNITS (if any)

Peter F. Kador, EI, LMOP, NIH
Frank Quinn, NCI
Jeffrey Gift, American University

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Membrane Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Energy minimization calculations and quantum mechanical calculations on compounds of biological and pharmacological interest continue to give insights into and explanations of their modes of behavior, resulting in clues to their pharmacophores.

The inhibition of the enzyme aldose reductase by a wide variety of compounds continues under investigation by QSAR techniques, as well as by energy minimization computations, quantum mechanics and stereochemical considerations. The pertinent factors have now been shown to include also bulk terms.

Energy minimization and quantum calculations have been carried out on the various conformations of colchicine and isocolchicine to correlate binding properties with the energies and structures of their conformations.

Various compounds showing promise against the AIDS virus are being systematically investigated to obtain structural and electronic properties which may help elucidate the mechanism of their action, and thus lead to improved forms. Energetic, structural and electronic properties of AZT have been obtained, as well as rotational barriers due to the base group.

The binding of analogs of colchicine has been investigated further by QSAR methods. In addition to partition coefficients, binding efficiency depends on free energy and molecular volumes and electronic properties.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29015-17-LCP

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Digital computer facilities for LCP and LMB

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

P.I.: W.H. Jennings, Jr.

Research Physicist

LCP-NIDDK

COOPERATING UNITS (if any)

Computer Systems Laboratory, DCRT: A.R. Schultz, Jr., J.I. Powell, D.C. Carpenter

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Membrane Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER:

1

1

CHECK APPROPRIATE BOX(ES)

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

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

The laboratory computer facility serving LCP and LMB was in routine operation during the reporting period. A change to an alternate configuration has begun with the upgrading of the host 11/70 CPU to an 11/84 with 2 Mb of memory. The magnetic tape drive was also replaced. Implementation of a workstation network in cooperation with CSL, DCRT has begun with the acquisition of a micro VAX and several SUN machines. This workstation network which uses Ethernet, UNIX and a network file server, will be developed as a parallel system and will not immediately impact operation of the existing facility. Closely related to the workstation network is a project to interconnect all terminals, personal computers and shareable peripherals. This system uses terminal servers on the Ethernet and will provide a bridge between the existing facility and the workstations.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01-DK-29016-13-LCP

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)
Macromolecular dynamics and assembly reactions

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

P.I. :	James Hofrichter	Research Chemist	LCP-NIDDK
Others:	William A. Eaton	Medical Officer	LCP-NIDDK
	Eric Henry	Research Chemist	LCP-NIDDK
	Lionel Murray	Staff Fellow	LCP-NIDDK
	Richard Lozier	Research Chemist	LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Spectroscopy and Structure

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.2

PROFESSIONAL:

2.2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Transient spectroscopy is used to study the kinetics of conformational changes in macromolecules subsequent to excitation with a pulsed laser. Changes in both the tertiary and quaternary structure of hemoglobin have been observed following the photodissociation of carbon monoxide from the hemes. The kinetics of ligand rebinding have also been studied in molecules which can be switched to the low-affinity 'T' state with ligands bound. The results show that the binding rate is reduced at least 30-fold in the photolytically produced deoxyhemoglobin molecule within ~20ns after the iron ligand bond is broken.

Transient spectroscopy has also been used to study the photoproduct cycle of bacteriorhodopsin, a molecule which pumps protons across the bacterial membrane. Our first studies have shown that the light-adaptation mechanism probably results from formation of multiple products in the photolysis step. In addition the data strongly suggest branching in the photocycle of the light-adapted molecule, in contrast to the conventional Br -> K -> L -> M -> O -> Br photocycle.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Spectroscopic investigation of membrane lipids and models

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

P.I.: Ralph G. Adams Research Physicist LCP-NIDDK

Other: Ira W. Levin Research Chemist LCP-NIDDK

COOPERATING UNITS (if any)

Sherwin Strauss (FDA)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS

1

PROFESSIONAL

1

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

Integrated intensity analysis of spectra obtained by temperature programmed Raman spectroscopy of artificial phospholipid membranes shows that thermal history (prior to spectroscopy) of specimens determines the course, rate and intensity of configurational alterations associated with the subtransition (crystal to gel state) of 12-18 carbon chain preparations. The more subtle spectral changes within the 2800-3100 cm^{-1} region (CH stretch), indicative of packing characteristics, we feel demonstrate that reorganization of packing occurs by domains rather than randomly.

Beginning efforts to understand the mechanics of lung surfactants in Adult Respiratory Distress Syndrome (ARDS), using the techniques above, show that a variety of surfactants, all effective, behave surprisingly differently considering that (theoretically) dipalmitoylphosphatidylcholine is the active ingredient common to all.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Theoretical studies on the dynamic aspects of macromolecular function

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

P.I.: A. Szabo

Research Chemist

LCP-NIDDK

COOPERATING UNITS (if any)

R. W. Pastor, R. Venable, Biophysics Laboratory, FDA

F. Kamp, LMB, NIDDK

M. Karplus, Dept. of Chemistry, Harvard University

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The results of a Brownian dynamics simulation of hydrocarbon chain in a membrane bilayer were used to analyze NMR relaxation experiments to yield information concerning the dynamics and ordering of lipids in vesicles. It was shown that the frequency dependence of the data does not arise from trans-gauche isomerizations or from axial rotation of the entire molecule. Quantitative agreement is found using a model in which fast axial rotations (~100ps) and large amplitude (with order parameter ~0.6) but slow director fluctuations (~10ns) are superimposed on the internal motions. This work supports a very fluid picture of the interior of the bilayer in contrast to the commonly accepted model in which crankshaft motions predominate. Using the stochastic theory of chemical reactions and the theory of first passage times, a simple analytic expression is derived for the distribution of delay times that has been observed in studies of the polymerization kinetics of sickle hemoglobin under conditions where the polymerization progress curves exhibit stochastic variation. The rate of homogeneous nucleation can be readily extracted from such experiments using this expression. This work constitutes a significant addition to the rather limited number of examples where contact can be successfully made between the stochastic theory of chemical kinetics and experiment. The influence of internal conformational dynamics on the electron transfer reaction between a donor and an acceptor was examined. The steady state flux resulting from the coupling of two multistate systems was shown to be identical to that calculated from a simple kinetic scheme involving only four states, if the effective rate constants of this reduced scheme are approximately defined in terms of the mean first passage times for moving between various points along the multistate cycles.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (90 characters or less Title must fit on one line between the borders)

Nuclear magnetic resonance: new methods and molecular structure determination

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

P.I. Ad Bax, Visiting Scientist, LCP-NIDDK

Others: Rolf Tschudin, Laura Lerner, Dominique Marion, Hong Ha, Neil Lewis, Guang Zhu

COOPERATING UNITS (if any)

Dennis A. Torchia, NIDR/LBR; B. Brooks, DCRT

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS

4.9

PROFESSIONAL

2.1

OTHER

2.8

CHECK APPROPRIATE BOX(ES)

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

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

The applicability of NMR methods for structure determination of proteins larger than 15 kD is being investigated. Approaches different from those applied to small proteins are needed to decrease spectral crowding associated with the increased number of hydrogens in the molecule. It has been demonstrated that perdeuteration of all non-exchangeable protons in the protein Staphylococcal Nuclease (18 kD) results in a large improvement of spectral quality of the exchangeable amide protons, permitting identification of the three alpha helical regions. Unambiguous site specific resonance assignments of the amide protons were then obtained by using a novel $^{13}\text{C}_1$ - ^{15}N double labeling approach.

An efficient method has been developed for measuring ^1H - ^1H J couplings in macromolecules where these couplings normally are not directly observable. The method has been applied to the DNA dodecamer $\text{d}(\text{CGCGAATTCGCG})_2$. Analysis of the measured couplings indicates that all deoxyribose sugars must undergo rapid conformational transitions between a minimum of two different geometries, a N-type and an S-type conformer. The fraction of time any deoxyribose exists in each conformation varies with its position in the dodecamer with, on average, the pyrimidine nucleotides having more N-character (10-20%) than the purines (0-10%).

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Conformation and dynamics of biological macromolecules

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

P.I. : Eric R. Henry	Research Physicist	LCP-NIDDK
Other: William A. Eaton	Medical Officer	LCP-NIDDK
James Hofrichter	Research Chemist	LCP-NIDDK
Lionel P. Murray	Staff Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

Bernard Brooks DCRT

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS

1

PROFESSIONAL

1

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

We have modified our transient absorption spectrometer to dramatically increase the speed of data acquisition and to improve the overall quality of the measured spectra. The instrument has also been interfaced to the advanced laboratory workstation (ALW) network in Building 2 to facilitate storage and analysis of data. We have extended our previous analysis, based on singular value decomposition, of time-resolved spectra measured on component I of trout hemoglobin over a wide range of temperatures. We have identified two early relaxations at low temperature which involve geminate ligand rebinding and deoxyheme spectral changes. The rates of these relaxations are temperature-dependent, but the rebinding yields and the amplitudes of the spectral changes are temperature-independent. These spectral changes have been assigned to tertiary structural changes in photolyzed subunits of the protein. A third relaxation with a temperature-dependent rate and involving a deoxyheme spectral change has also been identified, and we have assigned it to the quaternary structural change of the protein from the R to the T structure. The amplitude of this spectral change decreases as the temperature is increased, which we attribute to a decrease with increasing temperature of the physical size (and therefore spectroscopic effect) of the quaternary structure change of this protein. We are also performing a study of the hemoglobin tetramer using the techniques of molecular dynamics and perturbation free energy simulations.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Structural studies of AIDS proteins by NMR

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

P.I. :	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
Others:	Paul C. Driscoll	Visiting Fellow	LCP-NIDDK
	Ad Bax	Visiting Scientist	LCP-NIDDK

COOPERATING UNITS (if any)

Glaxo Institute of Molecular Biology, Geneva (Paul Wingfield); Genentech, San Francisco (Dan Capon); Genetics Institute, Boston; NCI (J.A. Berzofsky).

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS

2

PROFESSIONAL

2

OTHER

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

Work has been initiated on a number of structural problems related to proteins derived from the HIV virus. These include tat, art, 3'-orf, CD4, reverse transcriptase and proteins of the immune system. At this time, work is principally concentrated on obtaining sufficient material in a highly purified and active form for structural studies.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October, 1987 through September 30, 1988

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

Determination of Three-Dimensional Structures of Macromolecules in Solution by NMR

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

P.I. :	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK

Others:	Paul Driscoll	Visiting Fellow	LCP-NIDDK
	Paul Folkers	Geust Researcher	LCP-NIDDK

COOPERATING UNITS (if any)

Max-Planck Institute for Biochemistry, Martinsried, West Germany (M. Nilges, H. Oschkinat, T. Holak, C. Cieslar), Glaxo Institute of Molecular Biology, Geneva (Paul Wingfield).

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS

.75

PROFESSIONAL:

.75

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Work in this laboratory is focused on the determination of three-dimensional structures of macromolecules in solution by NMR. Methods are being developed to increase the precision with which structures can be determined, the molecular weight range of proteins that can be analyzed, and the efficiency of the computational methods used to determine the structures on the basis of the NMR data.

The solution structure of a small protein (43 residues) from sea anemone, called BDS, has been examined in detail. This particular protein lowers blood pressure without exhibiting cardiotoxic or neurotoxic properties, and in addition, has been shown to possess anti-viral activity against mouse hepatitis B virus. Complete sequential resonance assignments have been made and stereospecific assignments of most of the β methylene protons have been obtained at 600 MHz. A total of 513 interproton distance restraints and 50 torsion angle restraints have been derived from the measurement of nuclear Overhauser enhancements and three-bond coupling constants. This data is the basis of three-dimensional structure calculations using a hybrid distance geometry-dynamical simulated annealing approach. A total of 42 independent structures have been calculated. The atomic rms difference between the individual structures and the mean structure calculated by averaging their coordinates is 0.6 Å for the backbone atoms and 0.9 Å for all atoms.

Work is in progress on determining the solution structures of a number of other proteins. These include the DNA binding protein ner from phage Mu, human interleukin β , and native and mutant hirudin. In the case of both ner and interleukin β use is being made not only of $^1\text{H-NMR}$ but also of $^{15}\text{N-NMR}$ to help resolve spectral overlap.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October, 1987 to September, 1988

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

Design of Agents for Fluidizing HIV Virion Membrane

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

P.I.: Ira W. Levin Research Chemist LCP-NIDDK

Others: E. Neil Lewis Visiting Associate LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Fourier-transform (FT) Raman spectroscopic methods, using near infrared laser excitation, was developed for examining membrane related systems not amenable to conventional dispersive Raman techniques. The effects of macrolide polyene antibiotics, amphotericin A and amphotericin B (possible AIDS therapeutic agents) on model membrane systems were investigated using FT-Raman spectroscopic procedures. The two polyene antibiotics introduce interdigitated lipid phases in both cholesterol free and cholesterol containing membranes. The structural defects originating within the membrane from interdigitated lipid domains may be a critical factor in reducing HIV virion infectivity.

ANNUAL REPORT OF THE LABORATORY OF BIOORGANIC CHEMISTRY

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research of the Laboratory is directed towards the introduction of new concepts, techniques and agents for the elucidation of the molecular nature of mechanisms controlling cell functions. Specific focus is placed on i) Development of selective agonists/antagonists for receptors controlling cyclic nucleotide formation, phospholipid metabolism and ion channel function; ii) The relationship between ion transport, phospholipid turnover and cyclic nucleotide generation and the delineation of agents with specific effects on macromolecules involved in these systems. iii) The isolation and structure elucidation of biologically active natural products and definition of the basis of their activity. iv) Effects of agents on ion channels and the development of radioactive ligands for modulatory sites in such channels. v) The nature of enzymes involved in formation and inactivation of neurotransmitters, hormones, and other modulatory substances, in particular the enzymes, catechol-O-methyltransferase, monoamine oxidase, adenylate cyclase and phosphodiesterases. vi) the fundamental mechanisms by which drugs and environmental chemicals are transformed in the body with emphasis on oxidative metabolism by cytochrome P-450 systems to generate active oxide metabolites that interact with macromolecules such as DNA and are metabolized by further oxidation, by hydrolysis and by conjugation with glutathione.

Some of the milestones for the Laboratory are i) Introduction of the adenine-prelabeling technique for study of cyclic AMP generation in intact cells; ii) The steroidal alkaloid batrachotoxin as a selective activator of sodium channels. iii) Histronicotoxin as a noncompetitive blocker of acetylcholine receptor channels and potassium channels. iv) Pumiliotoxins as myotonic and cardiotoxic alkaloids acting through sodium channels to elicit phosphoinositide turnover. v) N⁶-Substituted adenosines and 8-phenyl and 8-cyclohexylxanthines as selective and potent adenosine receptor agonists and antagonists suitable as radioligands for binding studies and for definition of A₁ and A₂ classes of receptors. vi) Introduction of forskolin as a specific and widely useful activator of adenylate cyclase. vii) Fluoronorepinephrines and analogs as selective alpha and beta-adrenergic agonists. viii) Production of antibodies to catechol-O-methyl transferase and their use in studying localization of this key catechol-metabolizing enzyme. ix) Development of a cyclic quaternary amine, isoarecolone methiodide, with high potency and selectivity for nicotinic receptors. x) Definition of a relationship between receptor-activation of phosphoinositide breakdown; protein kinase C activation, and altered responses of cyclic AMP-generating systems. xi) Introduction of maitoxin as a general activator for phosphoinositide breakdown. xiii) Discovery of the NIH shift of aryl substituents during P-450 catalyzed phenol formation and demonstration of arene oxides as intermediates. xiv) Demonstration of oxidation-hydrolysis pathways that convert stereoselectively polycyclic aromatic hydrocarbons to ultimate diol epoxides that react with DNA. xv) Discovery and formulation of the bay-region theory, which is predictive of the pathway for formation of reactive carcinogenic metabolites from polycyclic aromatic hydrocarbons.

The laboratory accomplishes its mission both through its own resources and through extensive collaborations with other laboratories both at NIH, at Universities, Museums, and other institutes and in drug and chemical companies. Such collaborations can involve sharing of expertise on syntheses, isolations, analyses and biological testing and field work to obtain sources of new natural products.

SECTION ON PHARMACODYNAMICS

Pharmacologically Active Compounds from Amphibians and Other Sources

Alkaloids from Amphibians. Skin secretions from amphibians contain a wide range of biologically active substances, which can be tabulated under four categories: i) Biogenic amines; ii) Peptides; iii) Bufodienolides; iv) Alkaloids. Lipophilic alkaloids occur in skin of only five families of amphibians. Salamandines are steroidal alkaloids unique to the genus Salamandra of the family Salamandridae. They have potent local anesthetic activity. The two hundred dendrobatid alkaloids occur mainly in neotropical poison frogs of the family Dendrobatidae. These alkaloids have been assigned identifying code numbers and letters based on molecular weight and have a range of activities at various ion channels. The family is currently being subdivided, based in part on profiles of skin alkaloids, into the following genera: Phyllobates, which are unique in containing steroidal alkaloids of the batrachotoxin class; Dendrobates, Minyobates and Epipedobates, which contain a variety of piperidine and pyrrolidine-based alkaloids (histrionicotoxins, indolizidines of the 5-substituted, 5,8-disubstituted and 3,5-disubstituted subclasses, the pumiliotoxin-A class with pumiliotoxin, allopumiliotoxin and homopumiliotoxin subclasses, cis- and trans-decahydroquinolines, gephyrotoxins, 2,6-disubstituted piperidines, 2,5-disubstituted pyrrolidines, pyridylpiperidines, azatricyclododecenes, and amidines); Colostethus and Prostherapis, which do not contain lipophilic alkaloids. Dendrobatid alkaloids of the pumiliotoxin-A class also occur in South American toads of genus Melanophryniscus of the family Bufonidae and in Madagascan frogs of the genus Mantella of the family Ranidae and in Australian frogs of the genus Pseudophryne of the family Myobatrachidae. The Mantella frogs also contain histrionicotoxins, a decahydroquinoline, a 5,8-disubstituted indolizidine and alkaloids of unknown structure. The Pseudophryne frogs also contain a wide variety of prenyl pyrrolo[2,3-b]indole alkaloids, which are unique to these frogs. An erythro isomer of the dendrobatid alkaloid pumiliotoxin B has been found to occur along with pumiliotoxin B in Pseudophryne frogs. The cardiotoxic activity of this erythro-isomer is much lower than that of pumiliotoxin B.

A large number of alkaloids of unknown structure occur in extracts obtained from dendrobatid frogs. Many appear to be isomers of known alkaloids. Catalytic exchange and isomerizations are providing identification protocols for certain of these alkaloids. In addition, isolation and structure elucidation by nuclear magnetic resonance spectroscopy continues to provide definition of additional structures: The alkaloid 251F has been isolated from extracts of the Colombian frog Minyobates bombetes and appears to be a tricyclic alkaloid with a tertiary nitrogen and a CH_2OH , an isobutyl and a methyl substituent.

Skin extracts from one population of the poison-frog Dendrobates auratus contain a variety of alkaloids including histrionicotoxins, pumiliotoxin-A class

alkaloids, 2,5-disubstituted-cis-decahydroquinolines and 5-substituted-8-methyl-indolizidines. Three of the major alkaloids are cis-decahydroquinolines, whose structures based on nuclear magnetic resonance spectral analysis are 2,5-diallyl-cis-decahydroquinoline (cis-219A), 2-allyl-5-pent-2-en-4-ynyl-cis-decahydroquinoline (cis-243A) and 5-methyl-2-n-propyl-cis-decahydroquinoline (195A); the last identical with "pumiliotoxin C" previously isolated from the poison-frog Dendrobates pumilio. Alkaloids cis-219A and cis-243A differ in configuration from 195A at the C-2 position. Another poison-frog Dendrobates histrionicus was previously shown to produce nearly exclusively the corresponding trans-decahydroquinoline isomers of 219A and 243A. Both cis- and trans-219A inhibit binding of radioactive perhydrohistrionicotoxin to the nicotinic receptor channel complex and both block carbamylcholine-elicited sodium flux through the nicotinic receptor-channel of pheochromocytoma cells.

Skin extracts of the Panamanian poison frog Dendrobates speciosus contain at least thirty alkaloids. Eleven alkaloids were isolated by column chromatography in quantities sufficient for 2D-nuclear magnetic resonance spectral analysis, which in some cases confirmed their identity with alkaloids known from other species and in other cases led to assignment of structures. Pumiliotoxin 251D, pumiliotoxin A (307A), pumiliotoxin B (323A) and allopumiliotoxin 267A were identified as major constituents. N-oxides of 323A and 267A were also isolated. Indolizidines 195B and 223AB with 3-butyl-5-methyl and 3-butyl-5-propyl-substituents, respectively, were identified. The 5-substituents of the 8-methyl-indolizidines 207A and 235B' were assigned as $-(CH_2)_3CH=CH_2$ and $-(CH_2)_5CH=CH_2$, respectively; Indolizidine 235B' from D. speciosus is thus a positional double bond isomer of indolizidine 235B, previously isolated from a closely related poison frog, D. pumilio. A piperidine 241D was isolated and assigned the structure, cis-cis-2-methyl-6-nonyl-4-hydroxypiperidine. It is a very potent noncompetitive blocker of the nicotinic receptor-channel complex. Other noncompetitive blockers include histrionicotoxins 2,6-disubstituted pyrrolidines and 3,5-disubstituted indolizidines. A 5-substituted-8-methylindolizidine (205A) enhances binding of radioactive perhydrohistrionicotoxin at very low concentrations and then inhibits as its concentration is increased. Indolizidine 205A at low concentrations enhances sodium-flux through the nicotinic receptor-channel of pheochromocytoma cells, while having blocking action at higher concentrations.

Although dendrobatid frogs maintain levels of alkaloids after years in terrariums, captive-raised frogs of various species (Phylllobates terribilis, Dendrobates auratus, Dendrobates azureus, Dendrobates pumilio and Epipedobates tricolor) do not contain detectable amounts of alkaloids. The explanation of this remarkable finding is under investigation.

Synthesis of Piperidine 241D: A facile syntheses of 2,6-disubstituted-4-piperidines by condensation of an alpha, beta-unsaturated ketone, an aldehyde and ammonia has been developed. Condensation of 3-pentene-4-one, nonyl aldehyde, and ammonium acetate in methanol solvent yielded a mixture of cis and trans-2-methyl-6-nonyl-4-piperidones. The cis-isomer was catalytically reduced to yield racemic cis-cis-2-methyl-6-nonyl-4-hydroxypiperidine identical to the naturally occurring dendrobatid piperidine 241D. The method has been applied to the synthesis of other 2,6-disubstituted piperidines.

The Site of Action of Pumiliotoxin B on the Voltage-dependent Sodium Channel: Pumiliotoxin B (PTX-B), an alkaloid that has cardiotoxic and myotoxic activity, increases sodium influx in guinea pig cerebral cortical synaptoneuroosomes. In the presence of scorpion venom (Leiurus) or purified alpha-scorpion toxin, the PTX-B induced sodium influx is enhanced several-fold. PTX-B alone has no effect on sodium flux in N18 neuroblastoma cells but, in the presence of alpha-scorpion toxin, stimulation of sodium influx by PTX-B reaches levels comparable to that attained with the sodium channel activator veratridine. In neuroblastoma LV9 cells, a variant mutant that lacks sodium channels, neither veratridine nor PTX-B induces sodium fluxes in either the presence or absence of alpha-scorpion toxin. In synaptoneuroosomes and in N18 cells, the sodium influx induced by the combination of PTX-B and alpha-scorpion toxin is inhibited by tetrodotoxin and local anesthetics. PTX-B does not interact with two of the known toxin sites on the sodium channel, as evidenced by a lack of effect on binding of [3H]saxitoxin or [3H]batrachotoxin A benzoate to brain synaptoneuroosomes. Synergistic effects on sodium influx with alpha-scorpion toxin, beta-scorpion toxin, and brevetoxin indicate that PTX-B does not interact directly with three other toxin sites on the sodium channel: Thus, PTX-B appears to activate sodium influx by interacting with yet another site on the voltage-dependent sodium channel, a site that is coupled allosterically to sites for alpha-scorpion toxin, beta-scorpion toxin, and brevetoxin.

Structure Activity Relationships for Pumiliotoxins: The cardiotoxic activity of pumiliotoxin B (PTX-B) as assessed in guinea pig atrial preparations is markedly dependent on the nature of the 6-alkylidene side chain. Pumiliotoxin A (PTX-A), which differs from PTX-B only in lacking the 7'-hydroxy moiety, is much less active than PTX-B. Alteration in the configuration of the 6'- and/or 7'-hydroxy side chain moieties in synthetic isomers of PTX-B reduces activity, while the lack of such moieties or replacement with methoxy or ketone moieties in PTX-B or PTX-A analogues yields cardiodepressant compounds. PTX-B markedly stimulates phosphoinositide turnover in atrial and brain preparations and sodium influx in brain preparations, while analogues that are cardiac depressant or have low cardiotoxic activity have no or minimal effects on such parameters. It is suggested that activation of sodium channels and resultant stimulation of phosphoinositide breakdown play a role in the cardiotoxic activity of pumiliotoxin alkaloids.

Local Anesthetics: Effects on Batrachotoxin-elicited sodium flux and Phosphoinositide Breakdown: Local anesthetics inhibit the sodium influx and the inositol phosphate accumulation elicited by the sodium-channel activator batrachotoxin in guinea pig cortical synaptoneuroosomes. Inhibitory effects of local anesthetics on sodium influx correlate closely with inhibitory effects on binding of a tritiated batrachotoxin analog to sodium channels in synaptoneuroosomes. There is also a correlation between inhibitory effects on sodium influx and on inositol phosphate accumulation: Most local anesthetics inhibit sodium influx at concentrations similar to those required for inhibition of inositol phosphate accumulation. Indeed, euprocin, bupivacaine, lidocaine and certain analogs are nearly equipotent with respect to inhibition of sodium influx and inositol phosphate accumulation. Local anesthetics also inhibit inositol phosphate accumulation that is induced by carbamylcholine through both a tetrodotoxin-sensitive and a tetrodotoxin-insensitive pathway. Certain local anesthetics, such as dibucaine, inhibit the tetrodotoxin-sensitive pathway with higher potency than the tetrodotoxin-insensitive pathway, while others, such as

quinacrine inhibit tetrodotoxin-sensitive and insensitive pathways with equal potency. Diphenhydramine and chlorpromazine appear to inhibit carbamylcholine-elicited phosphoinositide breakdown through blockade of muscarinic cholinergic receptors rather than due to local anesthetic activity or to inhibitory effects on phospholipase C. Certain local anesthetics markedly stimulate incorporation of radioactive inositol into phosphoinositides.

SECTION ON PHARMACODYNAMICS

Pharmacology and Metabolism of Biogenic Amines and Related Compounds.

Studies on the structure of Catechol-O-methyltransferase (COMT). The major form of COMT (23000 Daltons, pI 5.2) has been isolated by a procedure using classical protein purification techniques, HPLC on reverse phase and ion exchange columns, and a final electro-elution from a Northern blot. The product migrated as a single protein on two dimensional PAGE and the amino acid composition was nearly identical to earlier preparations. Trypsin hydrolysis yielded several peptides which were isolated by HPLC. The major peptide with 19 amino acid residues had the following sequence AYVVPVAPIXTDKINAADYA. A cDNA probe has been synthesized and a search of several clones initiated.

Studies on the immunolocalization of COMT. The immunolocalization of COMT has been examined in the respiratory tract of rabbit and rat. The immunolocalization was compared to the localization of the adrenergic innervation and the neuroepithelial bodies of the respiratory tract. The neuroepithelial bodies are grouped as innervated neuroendocrine cells intercalated within the respiratory epithelium. In addition to a major innervation from the nodose ganglion of the vagus nerve, they are also innervated by adrenergic fibers. The adrenergic components were identified with a purified polyclonal antiserum to bovine tyrosine hydroxylase, the COMT-positive components with a polyclonal antiserum to the soluble form of rat liver COMT, and the neuroendocrine cells with a polyclonal antiserum to rat calcitonin gene related peptide. COMT-immunoreactivity was localized in the bronchial and bronchiolar subepithelial lamina propria. COMT-immunoreactivity also occurred in the tunica media of blood vessels and to a lesser extent in the airway smooth muscle. COMT-immunoreactivity disappeared where bronchioles transformed into terminal bronchioles and no reaction could be detected in alveoli. COMT-immunoreactivity was particularly dense beneath the grouped neuroendocrine cells. COMT is clearly correlated with the presence of adrenergic nerve fibers and located mainly extraneuronally in cells in the subepithelial lamina propria.

Studies on the substrate specificity and reaction mechanism of COMT. O-Methylation of optically active 3', 4'-dideoxynorlaudanosoline-1-carboxylic acids (DNLAC) afforded almost exclusively the 7-O-methylated acids. A similar almost exclusive O-methylation at the 7-hydroxyl obtained with the yellow quinone methine obtained from DNLAC at neutral or slightly alkaline pH by oxidative decarboxylation. Kinetic analysis of the enzymatic O-methylation of the S(+) and R(-)-DNLAC indicated a marked difference in the affinity for COMT. The Km for the S(+) form was 0.08 mM, while the value for the R(-) form was 0.50 mM. The Vmax values were nearly equal, 3.4 and 3.7 nanomoles/min/mg respectively, indicating that the relative turnover of the S(+) form was 6 fold greater than the R(-) form. Similar studies with the racemic DNLAC suggested

that the S(+) form was preferentially O-methylated even in the presence of equimolar amounts of the R(-)form. The nearly regiospecific O-methylation of both DNLAAC and the quinone methine essentially precludes the formation of 6-O-methylated isoquinolines of the 1-benzyl series as a major pathway in vivo.

Enzymatic O-methylation of norepinephrine (NE) by COMT selectively occurs of the *m*-phenolic group despite comparable acidities of its two phenolic groups. Since this selectivity is lost when the ethanolamine functionality is replaced by hydroxymethyl moiety, the amine moiety in the side chain must play a determining role in the regioselectivity of COMT. Previous studies with ring-fluorinated NEs and 5-fluorodopa have provided evidence that methylation occurs predominately at the phenolic group that ionizes more readily, indicating that nucleophilicity of the phenolic groups also plays an important role in the regioselectivity of COMT. A model for the catechol binding site of COMT that reconciles the role of the side chain functionality with that of the nucleophilicity of the phenolic groups is based upon the observed regioselectivity of COMT toward 5-FNE, which clearly indicates that the methyl group can be transferred to either *m*- or *p*-phenolic group with equal ease in the same binding mode. Contrary to previous models, we propose that the methyl group of S-adenosylmethionine is approximately equidistant from both the phenolic hydroxyl groups, so that direct nucleophilic attack can be mounted by either of the two phenolic groups.

Studies on the effects of fluorine substitution on the aromatic ring of biogenic amines. The systemic cardiovascular effects of the fluorinated catecholamine analogues, 6-fluoronorepinephrine (6-FNE) and 2-fluoronorepinephrine (2-FNE) were compared in the nembutal (R)-anesthetized rat following intravenous and intracerebral injection. The putative alpha-adrenergic agonist, 6-FNE, caused dose-dependent increases in blood pressure following intravenous injection that were inhibited by pretreatment with phentolamine. Similar injections of 2-FNE decreased blood pressure and increased heart rate. Propranolol pretreatment abolished the tachycardia and unmasked a pressor response, which could be abolished by combined pretreatment with propranolol and phentolamine. Intracerebral injections of 6-FNE, 2-FNE, and norepinephrine into the hypothalamus lowered blood pressure and heart rate. The time course of the response to 2-FNE was of shorter duration than was of 6-FNE or norepinephrine. The results support the contention that 6-FNE is a relatively selective alpha-adrenoceptor agonist in the doses used. The selectivity of 2-FNE is less and this analog apparently can stimulate both beta and alpha-adrenoceptors, in vivo, in doses commonly used to elicit cardiovascular actions.

Studies of the chemical and biological effects of fluorine substitution on the aromatic ring of catecholamines has now been extended to epinephrine. 2- and 6-fluoroepinephrines have been synthesized. Fluorine substitution on the 2- and 6-carbon of the aromatic ring alters the selectivity of epinephrine towards alpha- and beta-adrenergic receptors, similar in manner to the change in selectivity seen with norepinephrine. Thus, 2-fluoroepinephrine is a relatively selective beta-adrenergic ligand, while 6-fluoroepinephrine is a relatively selective alpha-adrenergic ligand. Fluorine substitution of epinephrine also can markedly increase potency at either alpha or beta-adrenergic receptors.

SECTION ON PHARMACODYNAMICS

Ion Channels, Receptors and Second Messengers in the Nervous System.

Maitotoxin: A Unique Agent for Activation of Phosphoinositide Turnover:

Maitotoxin (MTX), a high molecular weight polyether toxin isolated from a marine dinoflagellate, causes contraction of muscle and release of neurotransmitters and hormones from nerves and secretory cells. Such actions have appeared due to activation by MTX of voltage-dependent calcium channels and MTX has been proposed to be the most potent known activator of such channels with effects being seen at 100 pM. MTX has now been shown to be even more potent in stimulation or breakdown of phosphoinositides with effects being seen at 20 pM in a variety of cells as measured by the accumulation of inositol phosphates. Phosphoinositide breakdown results in the formation of two second messengers; inositol trisphosphate, which releases calcium from intracellular stores, and diacylglycerides, which activate protein kinase C.

MTX (mol wt. 3426) is an extraordinarily potent stimulant of phosphoinositide breakdown in the neuroblastoma hybrid NCB-20 cells. Maximal responses obtained at 0.25 - 0.5 ng MTX/ml, and result in an increase in [³H]inositol mono-, bis-, and tris-phosphates. Increased formation of [³H]inositol bis- and trisphosphate is observed as early as 15 sec after the addition of MTX. MTX-induced phosphoinositide breakdown in NCB-20 cells is not antagonized by organic (nifedipine, methoxyverapamil) or inorganic (Mn²⁺, Co²⁺, Cd²⁺) calcium channel blockers. However, the stimulation of phosphoinositide breakdown is completely eliminated in the absence of extracellular calcium. The results suggest that MTX either directly stimulates phosphoinositide breakdown in a calcium-dependent manner or acts indirectly through calcium channels insensitive to organic/inorganic calcium channel blockers.

Cell types in which MTX stimulates phosphoinositide breakdown include the following: neuroblastoma hybrid NCB-20 cells, pheochromocytoma PC12 cells, L-fibroblasts, basophilic leukemia RBL2H3 cells, human leukemia HL60 cells, insulinoma HIT cells, kidney 28A cells, glioma C6 cells, pituitary primary cultures and brain synaptoneuroosomes. In HL60 cells chemotactive peptide receptor-mediated phosphoinositide breakdown is inhibited by pretreatment with pertussis toxin, while MTX-mediated stimulation of phosphoinositide breakdown is not affected. Thus, it appears that MTX activation is not mediated through a receptor coupled to a pertussis-sensitive G protein. The effects of MTX on phosphoinositide breakdown are not inhibited by calcium channel blockers. However, removal of extracellular calcium results in a complete inhibition of MTX-induced effects. The threshold for the effects of 150 pM MTX on phosphoinositide breakdown is at 1 mM calcium for NCB-20 cells and 77 μM calcium for PC12 cells. The mechanism of MTX activation of phosphoinositide breakdown remains unclear. No direct effects on phospholipase C are seen in cell-free preparations. Since MTX is a general activator of phosphoinositide breakdown in a wide variety of cells, it represents a valuable tool to study this second messenger system.

In order to probe the value of MTX as a tool, the effects of MTX-elicited phosphoinositide breakdown on cyclic AMP accumulation in two cell lines was investigated. In these cells activation of protein kinase C with phorbol esters results in either inhibitory (NCB-20 cells) or stimulatory (PC12 cells) effects on the accumulation of cyclic AMP induced by receptor agonists or by a direct activator

of adenylate cyclase, forskolin. MTX does potentiate forskolin-induced accumulation of cyclic AMP in PC12 cells and does inhibit prostaglandin E_2 -induced accumulation of cyclic AMP in NCB-20 cells. The effects of MTX on accumulation of cyclic AMP are calcium-dependent and the concentrations of calcium required for maximal responses are the same as the ones required for maximal stimulation of phosphoinositide breakdown. MTX increases intracellular calcium in both cell lines, as measured by calcium-quin 2 fluorescence. But the effects of MTX on forskolin- and prostaglandin E_2 -mediated cyclic AMP accumulation are not mimicked by a calcium ionophore and are not blocked by nifedipine, a calcium channel blocker. Translocation of protein kinase C occurs after treatment with MTX in both cell lines; the protein kinase C activity and content are reduced in the cytosol and increased in membranes after exposure to either MTX or a phorbol ester. The results confirm previous studies on the heterogeneous (inhibitory and stimulatory) input of protein kinase C to cyclic AMP-generating systems performed with phorbol esters and demonstrate the utility of MTX as a unique tool for studies of systems that involve second messengers generated through stimulation of phosphoinositide breakdown.

MTX induced an exocytotic secretion of ATP from PC12 rat pheochromocytoma cells. The threshold for stimulation of secretion was at concentrations of about 2 ng/ml of MTX. Maximal release occurred at 40 ng/ml. MTX-induced ATP release required the presence of calcium in the extracellular medium and could be inhibited by nifedipine, a specific blocker of voltage-dependent calcium channels. In addition to the effects on ATP secretion from PC12 cells, MTX stimulated the breakdown of phosphoinositides, as measured by the accumulation of [3 H]inositol phosphates. Maximal stimulation of phosphoinositide breakdown was reached at only 0.5 - 1.0 ng/ml MTX. MTX at concentrations required to evoke ATP release (>2 ng/ml) had lesser or no effect on phosphoinositide breakdown. Although stimulation of phosphoinositide breakdown by MTX was dependent on extracellular calcium, it was insensitive to the calcium channel blockers nifedipine, D-600 and cobalt ions. The different concentration range required to elicit the responses and the differing sensitivity to calcium channel blockers indicate that MTX-evoked secretion and MTX-stimulated phosphoinositide breakdown are independent phenomena in PC12 cells.

Adenosine Receptors: Development of Xanthine Antagonists Selective for A_1 - or A_2 -Adenosine Receptors. A series of 1,3-dipropylxanthines were prepared with a variety of substituents at the 8-position. These included 8-aryl and 8-cycloalkyl groups. Polar carboxylate and carboxamide moieties were introduced as aryl substituents to increase water solubility. 1,3-dipropyl-8-[2-hydroxy-4-[(carboxymethyl)oxy]phenyl]-xanthine is a functionalized congener with high potency ($K_i = 37$ nM) and selectivity (54-fold) for A_1 -adenosine receptors. This congener was used for preparation of a series of other analogues, some with higher potency and some with higher selectivity. 8-Cyclopentyl- and 8-cyclohexyl-1,3-dipropylxanthines are both very potent ($K_i = 1-1.5$ nM) and selective for A_1 receptors, while 8-cycloalkylmethyl analogues are 10-fold less potent, but still very selective for A_1 receptors. 8-Piperidinyl and 8-pyrazinyl analogues have very low activities as adenosine receptor antagonists.

The effects of 8-phenyl and 8-cycloalkyl substituents on the activity of theophylline, caffeine, 1,3-dipropylxanthine, 1,3-dipropyl-7-methylxanthine, 3-propylxanthine and 1-propylxanthine at A_1 - and A_2 -adenosine receptors have been compared. An 8-phenyl substituent has little effect on the activity of caffeine or 1,3-dipropyl-7-methylxanthine at adenosine receptors, while markedly increasing

activity of theophylline, 1,3-dipropylxanthine, 3-isoamyl-1-isobutylxanthine, 1-methylxanthine and 3-propylxanthine. 8-Phenyl-1-propylxanthine is potent (K_i 20 nM) and selective for the A_2 receptor. A p-carboxy or p-sulfo substituent, which is introduced on the 8-phenyl ring to increase water solubility, in most cases decreases the activity and selectivity for the A_1 receptor. Among the 8-p-sulfo analogs, only 8-p-sulfophenyltheophylline and 1,3-dipropyl-8-p-sulfophenylxanthine are selective for the A_1 receptor. The remaining 8-p-sulfophenylxanthines are selective for the A_2 receptor. 8-Cycloalkyl substituents (cyclopentyl, cyclohexyl), markedly increase activity of caffeine and 1,3-dipropyl-7-methylxanthine at the A_2 receptor. 8-Cyclohexylcaffeine is potent (K_i 190 nM) and highly selective (147-fold) for the A_2 receptor. 8-Cyclohexyl-1,3-dipropyl-7-methylxanthine is more potent (K_i 85 nM) and 32-fold selective for the A_2 receptor. Such A_2 selectivity is in contrast to the marked A_1 selectivity of 8-cycloalkyltheophyllines and 8-cycloalkyl-1,3-dipropylxanthines.

Further Non-Xanthine Heterocycles as Adenosine Receptor Antagonists: A series of twelve 7-deaza-9-phenyladenines and of related 9-aryl-, 9-alkyl-, and 9-alkenyl-analogs and of 7-deaza-9-phenylhypoxanthines inhibit binding of [3 H]phenylisopropyladenosine to rat brain A_1 -adenosine receptors and antagonize activation of adenylate cyclase elicited by interaction of N-ethylcarboxamido-adenosine with A_2 -adenosine receptors in rat pheochromocytoma PC12 cell membranes. A subset of seven compounds, encompassing the range of major structural variations, antagonize inhibition of adenylate cyclase elicited by interaction of R-phenylisopropyladenosine with A_1 -adenosine receptors in rat fat cell membranes. 7-Deaza-9-phenyladenine has a K_i value of 3 μ M at the brain A_1 -receptor and a K_B value of 17 μ M at the PC12 A_2 -receptor and is thus about 5-fold more potent than theophylline at the former and nearly equipotent with theophylline at the latter. It has a K_B value of 4.6 μ M at the fat cell A_1 -receptor. The presence of methyl groups at the 7- and 8-positions reduce activity at all receptors several fold. Aryl substituents in a series of 7-deaza-7,8-dimethyl-9-phenyladenines do not have major effects on affinities for the brain A_1 - or the PC12 cell A_2 -adenosine receptors. The absence of the 9-phenyl substituent in the 7,8-dimethyl series reduce activity several fold, while replacement with arylalkyl ($-\text{CH}_2\text{C}_6\text{H}_4\text{F}$), alkyl ($-(\text{CH}_2)_5\text{CH}_3$) or alkenyl ($-\text{CH}_2\text{CH}=\text{CH}_2$) substituents has only modest effects on potency at the brain A_1 -receptor and the PC12 cell A_2 -receptor. 7-Deaza-7,8-dimethylhypoxanthine is nearly equipotent to the analogous 7-deazaadenine at the brain and fat cell A_1 -receptors, but is several fold more potent than the analogous 7-deazaadenine at the A_2 -receptor. 7-Deaza-7,8-dimethyl-9-(2,4-dibromophenyl)hypoxanthine is less potent than the analogous 7-deazaadenine at both the brain A_1 - and the PC12 cell A_2 -adenosine receptors. 7-Deaza-9-phenyl-7,8-benzohypoxanthine is the most potent of the present series of antagonists and is somewhat selective for the A_2 -adenosine receptor with a K_i of 0.9 μ M at the brain A_1 -receptor, a K_B of 1.4 μ M at the fat cell A_1 -receptor, and a K_B of 0.2 μ M at the A_2 -receptor.

3,5-Dimethylbenzo[1,2-c:5,4-c']dipyrazoles, optionally substituted in the 1-, 7-, and 8-positions, were synthesized from resorcinols. These compounds display affinity for adenosine A_1 (rat brain) and A_2 (human platelet) receptors. In addition, these compounds reverse contractions of guinea pig tracheal cylindrical segments induced by potassium chloride, histamine, acetylcholine, and 5-hydroxytryptamine, as well as reverse bronchospams induced by aerosolized histamine in the conscious guinea pig.

Caffeine and Theophylline Analogs: Selective In Vivo Effects as Adenosine Antagonists: The behavioral stimulant effects of xanthines, such as caffeine and theophylline, appear to involve blockade of central adenosine receptors. However, 3-isobutyl-1-methylxanthine (IBMX), a potent phosphodiesterase (PDE) inhibitor, produces behavioral depression. The effects of caffeine analogs on open field behavior of mice and potencies as antagonists of adenosine receptors and as inhibitors of three classes of brain PDE have been compared. 1,7-Dimethyl-3-propargylxanthine, 1,3,7-tripropargylxanthine, and 3,7-dimethyl-1-propargyl xanthine, which have high affinity for adenosine receptors and weaker activity as PDE inhibitors, all increase behavioral activity. In contrast, 1,3,7-tripropylxanthine, a more potent inhibitor of the brain calcium-independent (Ca-indep) PDEs than 1,3,7-tripropargylxanthine, produces behavioral depression, even though both analogues are potent adenosine receptor antagonists. 7-Benzyl-IBMX, an active receptor antagonist and selective inhibitor of a brain calcium-dependent (Ca-dep) PDE, produces a slight behavioral activation. Xanthines that are potent adenosine receptor antagonists and relatively weak inhibitors of the Ca-indep PDEs reverse the depressant effects of N⁶-cyclohexyladenosine, while xanthines, such as 1,3,7-tripropylxanthine, that are potent inhibitors of the Ca-indep PDEs, do not. The results suggest that the behavioral effects of xanthines may be determined primarily by relative activity as adenosine receptor antagonists and as inhibitors of brain-Ca-indep PDEs.

3,7-Dimethyl-1-propargylxanthine (DMPX), a caffeine analog that exhibits in vitro selectivity for A₂-adenosine receptors, compared to A₁-adenosine receptors, has now been investigated with respect to in vivo potency and selectivity. DMPX potently and selectively blocks the actions of the potent A₂ adenosine agonist 5'-N-ethylcarboxamidoadenosine (NECA) in DBA/2 mice, compared to blockade of the same responses elicited by the selective A₁-adenosine agonist N⁶-cyclohexyladenosine (CHA). DMPX is 57-fold more potent versus NECA-induced hypothermia than versus CHA-induced hypothermia and 11-fold more potent versus NECA-induced behavioral depression than versus CHA-induced behavioral depression. The hypothermia is mediated by peripheral receptors, based on blockade by 8-p-sulfophenyltheophylline (PSPT), while the behavioral depression is centrally mediated, based on lack of blockade by PSPT. DMPX is 28- and 15-fold more potent than caffeine in blocking peripheral and central NECA-responses, respectively. DMPX is equipotent with caffeine versus CHA-induced hypothermia and 2.5-fold more potent than caffeine versus CHA-induced behavioral depression. The motor stimulating potency of DMPX (ED₅₀ 10 µmol/kg) is slightly greater than caffeine.

SECTION ON OXIDATION MECHANISMS

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites.

Previous annual reports from this Section have described a systematic approach to the study of the cytotoxicity, mutagenicity and carcinogenicity of several polycyclic aromatic hydrocarbons. Briefly, these studies have consisted of i) synthesis of as many known and potential oxidative metabolites as was possible, ii) study of the metabolism of the hydrocarbons with these authentic standards in hand, iii) testing these compounds for cytotoxic and mutagenic activity with bacterial and mammalian cells both in the presence and in the absence of added drug metabolizing systems such as cytochrome P-450 and epoxide hydrolase, iv) identification of products formed by covalent addition of these

reactive metabolites to biological macromolecules such as DNA and v) evaluation of the carcinogenicity of the synthesized metabolites in several animal models. These studies provided evidence which indicated that bay-region diol epoxides, formed by enzymatic epoxidation of trans-dihydrodiols, are the most potent carcinogenic metabolites of these hydrocarbons. We formulated the "bay-region" theory which predicts that diol epoxides that have the epoxide group in the bay region of the hydrocarbon will be the most chemically reactive and presumably biologically active diol epoxides from hydrocarbons that are tumorigenic. To date studies from our laboratory as well as several other laboratories around the world have either proved or implicated bay-region diol epoxides as ultimate carcinogens formed from benzo(a)pyrene, benz(a)anthracene, benz(c)acridine, 7-methylbenz(a)anthracene, 7-methylbenz(c)acridine, benzo(b)fluoranthene, 7,12-dimethylbenz(a)anthracene, 3-methylcholanthrene, dibenz(a,h)anthracene, two dibenzpyrenes, chrysene, 5-methylchrysene, benzo(c)phenanthrene, and certain methylated cyclopentaphenanthrenes. The theory has stimulated considerable research in the field, all of which has supported our initial concepts. To date, there are no significant known exceptions.

Aspects of hydrocarbon-induced carcinogenesis which the bay-region theory made no attempt to take into account include effects of the relative and absolute stereochemistry of ultimate carcinogens, as well as the conformation of the hydroxyl groups on the benzo ring of the diol epoxides. Many of our current efforts address these questions. Studies of the dihydrodiols and resultant bay-region diol epoxides formed from benz(a)anthracene as well as phenanthrene and chrysene by liver microsomes have shown that these molecules are all superimposable with the corresponding benzo(a)pyrene metabolites when their bay regions are aligned. In the benzo(a)pyrene case, only one of four stereoisomeric bay-region 7,8-diol-9,10-epoxides exhibits strong tumorigenic activity, namely the predominant metabolically formed isomer. Tumor studies have shown that the related stereoisomer (R,S-diol-S,R-epoxide) is also the most active form from chrysene, benz(a)anthracene and benzo(c)phenanthrene. Results of the present studies are suggestive that there is a highly enantioselective site with which these carcinogens interact within the cell. Studies are in progress that will further define the steric constraints of the active site of cytochrome P-450c, the principal oxidative enzyme responsible for the conversion of polycyclic aromatic hydrocarbons to ultimate carcinogens.

We had previously observed that diol epoxide-1 isomers (in which the benzylic hydroxyl group is cis to the epoxide oxygen) normally exhibit little or no tumorigenic activity. In the absence of unusual steric or electronic factors, these isomers prefer the conformation in which the hydroxyl groups are pseudoaxial, whereas the diol epoxide-2 isomers (with the benzylic hydroxyl group trans to the epoxide oxygen) normally prefer the conformation with pseudoequatorial hydroxyl groups. The carcinogenic (R,S)-diol (S,R)-epoxides are of the latter type (diol epoxide-2). We proposed that pseudoaxial orientation of the hydroxyl groups (as in diol epoxides-1) might inhibit tumorigenic activity. This suggestion was supported by the observation that diol epoxide-2 from benzo(e)pyrene, whose conformation is unusual in that the hydroxyl groups are pseudoaxial, has extremely low tumorigenic activity. Furthermore, a diol epoxide-1 isomer from benzo(c)phenanthrene, whose hydroxyl groups prefer the pseudoequatorial conformation, "abnormal" for diol epoxide-1, exhibited substantial tumorigenic activity on mouse skin. A further test of the hypothesis that pseudoaxial hydroxyl groups inhibit tumorigenic activity was designed using 6-fluorobenzo(a)pyrene (6-FBP) diol epoxides. 6-FBP diol epoxide-2 was expected to prefer the unusual conformation with pseudoaxial hydroxyl groups, although closely

resembling the carcinogenic unfluorinated analogue in overall molecular dimensions. This prediction regarding conformation has now been shown to be the case. Studies of the effects of the fluorine substituent on the solution chemistry of the 6-FBP diol epoxides as well as tumor studies are complete.

Chemistry and Metabolic Formation of Arene Oxides and Their Derivatives. Because of our interest in the stereoselectivity of metabolism and tumorigenic activity of polycyclic aromatic hydrocarbon derivatives, the Section has a strong commitment to the development of methods for the determination of absolute configuration of metabolites from these hydrocarbons, as well as the synthesis of these metabolites in optically pure form.

Several years ago this laboratory developed a highly efficient procedure for the facile synthesis of reactive benzo-ring arene oxides. The immediate precursors were 1-acetoxy-2,4-dibromo derivatives on 1,2,3,4-tetrahydrobenzo rings. Recently, we have observed that these precursors from selected hydrocarbons (e.g., triphenylene, benzo(e)pyrene, benz(a)anthracene and dibenzanthracenes) form not only the desired arene oxides but also novel oxepins in which the aromatic portion is annellated to the 2,3-bond of the oxepin. In addition, the arene oxides readily photoisomerize to the same oxepins with room light. Those hydrocarbons for which oxepin formation occurs can be predicted from PMO calculations of differences in resonance energy between the oxepins and arene oxides.

Metabolism of triphenylene by liver microsomes from control, phenobarbital (PB)-treated rats and 3-methylcholanthrene (MC)-treated rats as well as by a purified system reconstituted with cytochrome P-450c in the absence or presence of purified microsomal epoxide hydrolase was examined. Control microsomes metabolized triphenylene at a rate of 1.2 nmol/nmol of cytochrome P-450/min. Treatment of rats with PB or MC resulted in a 40% reduction and a 3-fold enhancement in the rate of metabolism, respectively. Metabolites consisted of the *trans*-1,2-dihydrodiol as well as 1-hydroxytriphenylene, and to a lesser extent 2-hydroxytriphenylene. The (-)-1R,2R-enantiomer of the dihydrodiol predominated (70 to 92%) under all incubation conditions. Incubation of racemic triphenylene 1,2-oxide with microsomal epoxide hydrolase produced dihydrodiol, which was highly enriched (80%) in the (-)-1R,2R-enantiomer. Experiments with ¹⁸O-enriched water showed that attack of water was exclusively at the allylic 2-position of the arene oxide, indicating that the 1R,2S-enantiomer of the oxide was preferentially hydrated by epoxide hydrolase. Thiol trapping experiments indicated that liver microsomes from MC-treated rats produced almost exclusively (>90%) the 1R,2S-enantiomer of triphenylene 1,2-oxide whereas liver microsomes from PB-treated rats formed racemic oxide. The optically active oxide has a half-life for racemization of only ~20 s under the incubation conditions. This study may represent the first attempt to address stereochemical consequences of a rapidly racemizing intermediary metabolite.

Kinetics and Mechanisms of Reactions of Diol Epoxides and Related Compounds in Aqueous Solution. The rates of reaction of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (DE-2) in 1:9 dioxane-water (v/v) solutions containing the 2', 3', and 5'-monophosphate derivatives of guanosine, adenosine, and cytidine have been determined. From plots of k_{obsd} vs. concentration of nucleotide at several different pH values, it could be concluded that the monohydrogen phosphate ionization state was responsible for catalyzing the hydrolysis of DE-2. It was therefore assumed that the monohydrogen phosphate group acted as a general acid catalyst in the epoxide hydrolysis reaction. The second-order rate constants for the general acid catalyzed

hydrolysis of DE-2 by the nucleotides listed above were determined. The 2'- and 5'-monophosphates of guanosine and adenosine are better general acid catalysts than the corresponding 3'-isomers, although the 3'-isomers are stronger acids. The guanosine and adenosine nucleotides are better catalysts than the cytidine monophosphates. It was concluded that not only does the monohydrogen phosphate group act as a general acid but the secondary interactions of a stacking nature between the aryl group of DE-2 and the base are important for catalytic effectiveness.

Solvolysis reactions of most highly reactive benzylic epoxides studied to date exhibit simple biphasic pH-rate profiles consisting of a hydronium-ion dependent (k_H) and a pH-independent (k_O) region. In contrast, the pH-rate profile (extrapolated to zero buffer concentration) for the solvolysis of precocene I 3,4-oxide (3,4-epoxy-2-(2-dimethyl-7-methoxy-2H-benzo(b)pyran) to diols at 25 °C in 1:9 dioxane-water, at ionic strength 0.1 M (NaClO₄), is more complex. Besides the transition from k_H to k_O at pH ca. 7, there is a break at pH ca. 9, such that the rate constants observed above this pH fall below those predicted from the pH-independent rate constant (k_O) determined at lower pH values. Furthermore, the observed rate constants for general acid catalysis of the solvolysis by buffers at pH 8-9.3 show a nonlinear dependence on buffer concentration. Both these observations are consistent with a stepwise mechanism with a change in rate determining step from formation of a carbocation intermediate (at low pH and low buffer concentrations) to capture of the carbocation by solvent (at higher pH and high buffer concentrations). In the presence of 0.2 M acetylhydrazide, CH₃C(O)NHNH₂, at pH 8.5, approximately 80% of the reaction products corresponded to acetylhydrazide adducts of the epoxide, although the rate of reaction was increased by less than 10% by the added nucleophile. This is consistent with the trapping of an intermediate subsequent to the rate-determining step. Products of the reaction at low pH are the 3,4-diols resulting from addition of water trans and cis to the epoxide oxygen, whereas at higher pH a substantial fraction (20-75%) of the products corresponds to a ketone produced presumably by a hydride-ion shift. On the basis of kinetic and product data, we propose that this ketone is formed from the epoxide by a process that does not share a common intermediate with the stepwise formation of diols. The kinetic deuterium isotope effect for the migrating hydrogen was estimated to be ca. 4.0.

Biological Activity of Oxygenated Metabolites. Tumorigenic activities of the (7R,8S,9S,10R)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro derivatives of benzo(a)pyrene [(+)-BP diol epoxide-2] and 6-fluorobenzo[a]pyrene (6-FBP diol epoxide-2) were evaluated in newborn CD-1 mice. A total dose of 14 nmol of either diol epoxide was administered to preweaning mice, and tumorigenic activity was determined when the mice were 32 to 36 weeks old. At the termination of the study, 13% of solvent-treated control mice had developed lung tumors with an average of 0.19 tumor per mouse. No other tumors were observed in control animals. (+)-BP diol epoxide-2 induced pulmonary tumors in 60% of the mice with an average of 1.9 tumors per mouse, and 14% of the male mice developed hepatic tumors with an average of 0.18 tumor per mouse. In contrast, 6-FBP diol epoxide-2 had no significant tumorigenic activity at the 14-nmol dose. Although both bay-region diol epoxides have the same absolute configuration, (7R,8S,9S,10R), the hydroxyl groups of (+)-BP diol epoxide-2 prefer the pseudoaxial conformation whereas the hydroxyl groups of 6-FBP diol epoxide-2 prefer the pseudoaxial conformation. These tumorigenicity results are the first direct demonstration that the conformation of the hydroxyl groups in a bay-region diol epoxide, in addition to the documented effect of absolute configuration, is an important determinant in the tumorigenic activity of these ultimate carcinogens.

Potential Antitumorigenic Agents. A recent goal of research in the Section has been the identification of compounds capable of blocking the biological activity of bay-region diol epoxides through their chemical inactivation. Several years ago, ellagic acid, a naturally occurring plant phenol, was identified by us as a potent inhibitor of the mutagenic action of benzo(a)pyrene diol epoxide-2, and was shown to inactivate the diol epoxide in aqueous solution by forming covalent ether adducts. Subsequent studies indicated that ellagic acid had a moderate inhibitory effect on the tumorigenicity of this diol epoxide on mouse skin, but was inactive against the parent hydrocarbon. The inability of ellagic acid to inhibit significantly the tumorigenicity of benzo(a)pyrene may possibly result from its poor availability in target cells. Studies continue in this area of tumor inhibition. Several fatty acids as well as a group of lipophilic plant products with acidic hydroxyl groups are under investigation.

Covalent Modification of DNA by Diol Epoxides. In a previous annual report we described the isolation and complete structural characterization of the deoxyguanosine (dG) and deoxyadenosine (dA) adducts formed upon covalent modification of DNA in vitro by the four optically active benzo(c)phenanthrene 3,4-diol 1,2-epoxides. As part of an ongoing program to determine relationships between chemical structure, tumorigenicity and the extent and type of DNA adduct formation by these diol epoxides, we have undertaken similar studies with the optically isomeric 3,4-diol-1,2-epoxides derived from dibenz(a,j)anthracene (DBA) and benz(a)anthracene (BA). As previously reported for the cases of benzo(a)pyrene (BP) and benzo(c)phenanthrene (BPh), the rates of disappearance from aqueous solution of the diol epoxides derived from BA and DBA are markedly accelerated in the presence of DNA. At neutral pH these rates are ca. 20-100 fold faster in the presence of 1 mg/ml of calf thymus DNA than in aqueous buffers alone. The overall reaction consists of a combination of hydrolysis of the diol epoxides to tetraols and covalent DNA adduct formation. The efficiency of DNA adduct formation relative to hydrolysis is strongly dependent on the nature of the hydrocarbon. As previously reported 55-75% of the total diol epoxides is covalently bound to DNA in the case of BPh; present results indicate that for BA and DBA the efficiency of DNA adduct formation relative to hydrolysis is only 10-24% and 3-10%, respectively. Interestingly, for both DBA and BA, the (4R,3S,2S,1R)-diol epoxide-2 enantiomer exhibits the greatest extent of DNA adduct formation, although the magnitude of the difference between this and other isomers is only 2-3 fold. For those diol epoxides for which tumor data are available, this optical isomer has been shown to be the most tumorigenic, as well as the predominant isomer formed metabolically. Preliminary characterization of the nucleoside adducts formed upon reaction of calf thymus DNA in vitro with each of the optically active diol epoxides from BA and DBA, followed by enzymatic digestion, has permitted identification of the purine base component of each of the chromatographically separable nucleoside adducts. Although the BPh diol epoxides had exhibited a general preference for adduct formation with deoxyadenosine (dA), relative to deoxyguanosine (dG) residues (55-86% of total adducts formed), the diol epoxides derived from both BA and DBA exhibit less of a preference for reaction with dA (22-37% of BA adducts and 20-34% of DBA adducts). An even stronger preference for dG adduct formation has been observed for the BP diol epoxides. Detailed spectroscopic studies of the DBA and BA adducts, currently in progress, will permit unequivocal assignment of their structures and of the stereochemistry (cis or trans) of addition of the nucleoside bases to the epoxide group in each case.

There is no obvious correlation between gross binding efficiency to DNA and the tumorigenic response elicited by diol epoxide-2 diastereomers derived from different

hydrocarbons. For racemic diol epoxide-2 diastereomers whose tumorigenicity is known, the following order of biological activity is observed: BPh > BP > BA. In contrast, the overall efficiency of binding of these diol epoxides to DNA in vitro follows the order: BPh > BA > BP. Similarly, comparison of tumorigenicity with ratios of total dG to total dA adduct formation for the three hydrocarbons reveals no clear trend. More refined structural studies as to damage at specific sites on DNA may be required before clear relationships with tumorigenicity emerge.

SECTION ON PHARMACODYNAMICS

Nicotinic and Muscarinic Acetylcholine Receptor Agonists.

Isoarecolone methiodide (ISO) has been shown to be a highly potent agonist in nicotinic assays (usually 10-50 times more potent than carbamylcholine and several times more potent than acetylcholine, depending on the assay). ISO causes much less neuromuscular blockade, primarily due to desensitization, than do other standard nicotinic agonists. 3,4-Dihydro ISO is another potent ligand in the Torpedo and neuromuscular assays. Isoarecolone HCl produced nicotine-like behavioral effects in rats. Interestingly, ISO shows affinity for both nicotinic and muscarinic (M1) receptors, similar to the neurotransmitter acetylcholine.

SECTION ON OXIDATION MECHANISMS

Mechanistic Enzymology of HIV Proteins, An Approach to Rational Drug Design.

This project is designed to elucidate the mechanism of action of HIV enzymes by the use of affinity labeling agents and/or mechanism based inhibitors. The implications for rational design of anti-HIV drugs are self-evident. Even if the specific compounds investigated do not have anti-HIV activity per se, knowledge gained concerning the active sites of HIV enzymes is expected to provide valuable guidance in the design of the next generation of mechanism-based inhibitors. The development of a rapid, continuous optical assay for reverse transcriptase will facilitate greatly the screening of potential inhibitors for this important class of viral enzymes.

Several nucleoside and peptide analogues have been prepared, and these have been tested for anti-HIV activity by the laboratory of Dr. Samuel Broder, NCI. 2',3'-Epoxyadenosine, whose triphosphate is a known inhibitor of *E. coli* DNA polymerase I, and 3'-ketothymidine exhibited no anti-HIV activity in cell culture. Experiments are underway to determine whether the triphosphates of these compounds may be inhibitors of reverse transcriptase. Three new compounds that are potential aspartyl protease inhibitors have been prepared. Two of these are diastereomeric alcohols, methyl N-(2-furylacryloyl)-4-amino-3-hydroxy-5-phenyl-pentanoates (derived from L-phenylalanine), which differ in absolute configuration at C-3; the third compound is the ketone obtained upon oxidation of the 3-hydroxyl group of these alcohols. These compounds are related to statine, a component of the potent aspartyl protease inhibitor, pepstatin. Both diastereomeric alcohols exhibited no antiviral activity at concentrations of 1 or 10 micromolar, and were highly cytotoxic at 100 micromolar.

Three natural products derived from South Pacific marine sponges, mycalamide

A (S 60025-A), mycalamide B (SP60025-B) and SP50070, whose structure is presently unknown, were tested for anti-HIV activity at concentrations between 0.0001 and 0.1 microgram/ml. No anti-HIV activity was detected at low concentrations, and substantial cytotoxicity prevented the assessment of antiviral effects at higher concentrations.

SECTION ON OXIDATION MECHANISMS

Mass Spectrometry of Drugs, Metabolites and Natural Products.

Techniques developed during the last two years include deuterium exchange electron impact-mass spectrometry and liquid chromatography-mass spectrometry, special techniques for mass spectrometry on non-volatile and high molecular weight compounds, Ion Trap Self-Chemical Ionization and tandem (MS/MS) ion trap mass spectrometry, adduct identification in methanolysis products of arene oxides and counter current chromatography mass spectrometry. A large number of collaborations are in progress where mass spectrometry is key to the identification of new biologically active compounds from both synthetic and natural sources.

Marine Natural Products: Bioassay - directed analysis of a New Zealand sponge of the genus Mycale (family Mycalidae, order Poecilosclerida) by Drs Blunt and Munro (Univ. Canterbury) led to the isolation of mycalamide, a compound with potent in vivo antitumor and antiviral properties. Its mass spectrum showed similarities to a previously reported compound (Pederin). Further mass spectrometry linked with a combination of one- and two-dimensional NMR techniques allowed the determination of the structure. The compound is presently under clinical study.

A second material, a sulfoxide, was isolated by Drs. Blunt and Munro from the New Zealand ascidian Riterella sigillinosides and found to be identical to the sulfoxide produced synthetically from the oxidation of eudistomin K. Its structure was determined by a combination of mass spectrometry and NMR techniques. The compound has antiviral activity.

Frog Alkaloids: Amphibian skin has proven a rich source for a variety of unique alkaloids. As part of an investigation of the alkaloids in different species, an unusual prenyl pyrrolo[2,3-b]indole ester was isolated from an Australian frog Pseudophryne coriacea. A combination of gas chromatographic and desorption probe techniques allowed the assignment of a tentative structure. After careful purification and further mass spectrometry coupled with NMR techniques, the structure was ratified.

Plasma Desorption Mass Spectrometry: Plasma desorption mass spectrometry is a method particularly suited to the analysis of difficult compounds and has found a wide range of uses. The instrument at NIH is located in LC, NHLBI, but has been designed, built and run as a collaborative effort with Dr. Fales of LC. Very few of these mass spectrometers exist and so the request for analyses are wide ranging. It was found to be invaluable in the characterization of "The brain's own clonidine..." and in the synthesis and purification of two tetranuclear manganese complexes which are models for the photosynthetic water-splitting system in plants.

Countercurrent Chromatography - Mass Spectrometry: Countercurrent chromatography developed by Dr. Ito in LTD, NHLB has suffered from a lack of detection techniques. Following a similar approach to liquid chromatography-mass spectrometry, a countercurrent chromatography-mass spectrometry system has been developed in collaboration with LTD. Two plant extracts were separated by countercurrent chromatography and their mass spectra determined. The technique allows for very rapid separations and analyses.

Polycyclic aromatic hydrocarbon epoxides: As part of the routine mass spectrometry development/service to the Section on Oxidation Mechanisms, LBC, a total of approximately 250 samples related to polycyclic aromatic hydrocarbon epoxides were run during the past year.



NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31100-23 LBC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

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

P.I.	J. W. Daly	Chief	LBC, NIDDK
Others:	C.R. Creveling	Research Chemist	LBC, NIDDK
	T. Spande	Research Chemist	LBC, NIDDK
	M. Edwards	Chemist	LBC, NIDDK
	F. Gusovsky	Senior Staff Fellow	LBC, NIDDK
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	Y. Nishizawa	Guest Worker	LBC, NIDDK

COOPERATING UNITS (if any)

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LABORATORY of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

5.5

PROFESSIONAL:

5.0

OTHER:

0.5

CHECK APPROPRIATE BOXES)

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

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

Natural products have provided a wide range of biologically active agents, many of which have unique profiles of pharmacological activity and therapeutic potential. Over two hundred alkaloids have been identified in extracts from amphibian skins. These include batrachotoxins, which are potent activators of sodium channels, histrionicotoxins, which are noncompetitive blockers of nicotinic receptor channel complexes and of potassium channels, and pumiliotoxins, which have myotonic and cardiotoxic activity due to inhibitory effects on closing of sodium channels. Pumiliotoxin B acts at a specific site on the sodium channel to increase sodium flux and augments the effects of other sodium channel agents, such as the scorpion toxins and brevetoxins. Structure activity relationships indicate a stereoselective interaction with the side chain hydroxy groups of the pumiliotoxin alkaloids. Local anesthetics inhibit the stimulatory effects of batrachotoxin on both sodium flux and phosphoinositide breakdown in brain synaptosomes. In addition, local anesthetics stimulate incorporation of inositol into phosphoinositides. A 5-substituted-8-methylindolizidine markedly enhances and then at higher concentrations inhibits binding of radioactive perhydrohistrionicotoxin to the nicotinic receptor-channel complex. It has agonist activity at nicotinic receptors of pheochromocytoma cells. Other dendrobatid alkaloids, such as both the cis- and trans-2,5-disubstituted decahydroquinolines, the 3,5-disubstituted indolizidines, the 2,5-disubstituted pyrrolidines, the 2,6-disubstituted piperidines and a 2,6-disubstituted-4-hydroxypiperidine are potent inhibitors of binding of perhydrohistrionicotoxin and have noncompetitive antagonist activity at nicotinic receptors of pheochromocytoma cells. The biological activity of trace alkaloids from dendrobatid frogs, such as the azatricyclododecenes, the amidines and the homopumiliotoxins, and of the tricyclic indole alkaloids from myobatrachid frogs remain unknown. One amidine alkaloid is a potent analgetic.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31101-20 LBC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Pharmacology and Metabolism of Biogenic Amines and Related Compounds

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

P. I.:	C.R. Creveling	Research Chemist	LBC, NIDDK
Others:	J.W. Daly	Chief	LBC, NIDDK
	F. Gusovsky	Senior Staff Fellow	LBC, NIDDK

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NIDDK - FDA

LABORATORY
Laboratory of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

The chemistry, biochemistry, physiology, and pharmacology of biogenic amines, their amino acid precursors and metabolic products, and various synthetic derivatives have been investigated. The areas of interest include catechol-O-methyltransferase (COMT) and the effects of fluorine substitution on the properties of biogenic amines and amino acids. These include 1) development of improved methodology for the purification of COMT, utilizing high pressure liquid chromatography, 2) enzymatic hydrolysis, isolation, and determination of the primary sequence of peptides derived from COMT, 3) preparation of oligonucleotides from COMT-specific peptide sequences and the identification clones from rat liver. 4) immunohistochemical localization of COMT in normal, and malignant tissues of rodent and man at the light and electron microscopic level. The tissues include: a) the gastrointestinal track of rat and man, b) the ovary, uterus and oviduct in virginal and pregnant golden hamster, c) hormonally sensitive and insensitive human breast tumors, d) neuroepithelial bodies associated with adrenergic innervation of lung, e) macrophages from rat ovary, f) fibroblasts present in dental pulp, and g) normal human skin, premelanotic nevi, and malignant melanoma. 5) the substrate specificity and reaction mechanism of COMT including a) O-methylation of (S)- and (R)-dideoxynorlaudanosoline-1-carboxylic acids, and a series of related non-catecholic quinone methines as possible precursors of endogenous mammalian opiates, b) the effects of substitution of fluorine at the 2,5, and 6 carbons on the aromatic ring of epinephrine on the affinity and reaction rates with COMT, c) the effects of disubstitution of fluorine at the 2,5- and 2,6-carbons of norepinephrine on the affinity and reaction rates with COMT, d) the effects of fluorine substitution on the aromatic ring of dihydrophenylserines on the interaction parameters of COMT, 6) the alpha- and beta-adrenergic properties of fluorine substituted catecholamines and related compounds, 7) the mechanism of toxicity of 6-fluoro-2,6-difluorophenylalanine and 6-fluoro- and 2,6-difluorotyrosine in cultured pheochromocytoma and melanoma cell lines.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31102-17 LBC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Ion Channels, Receptors and Second Messengers in the Nervous System

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

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COOPERATING UNITS (if any)

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LAB BRANCH
Laboratory of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

4.3

PROFESSIONAL:

2.8

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

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

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

Physiological functions are mediated in different cells by a variety of second messengers, including cyclic AMP and cyclic GMP. Ions such as calcium, sodium and magnesium can serve after translocation through ion channels or by transport proteins as second messengers to cause activation of release processes, contractile proteins, adenylate and guanylate cyclase, phosphodiesterases, protein kinases, phospholipases, ATPases and other enzymes. Enzymatic hydrolysis of phospholipids can also generate second messengers, such as arachidonate, which serves as a precursor of prostanoids, diacylglycerides, which serve as activators of protein kinase C, and inositol phosphates, which serve as mobilizers of internal calcium ions. Receptors of various types serve to modulate ion channels and generation of second messengers. Adenosine stimulates cyclic AMP formation through an A₂ receptor and inhibits cyclic AMP formation through an A₁ receptor and also can regulate calcium and potassium channels and phosphoinositide breakdown. Maitotoxin (MTX), a high molecular weight polyether toxin, is a very potent stimulant of phosphoinositide breakdown in a wide range of cell types. This effect occurs at lower concentrations of MTX than those required to activate calcium channels. Blockade of calcium channels has no effect on MTX-evoked stimulation of phosphoinositide breakdown. But removal of extracellular calcium completely blocks MTX-induced effects. MTX-induced phosphoinositide breakdown has similar effects to phorbol esters on translocation of protein kinase C and on responsiveness of cyclic AMP-systems in two cell lines. Further selective xanthine antagonists for adenosine receptors were developed with 8-cyclohexyl-1,3-dipropylxanthine being 130-fold selective for A₁ receptors and 8-cyclohexylcaffeine being 150-fold selective for A₂ receptors. Certain 9-phenyl-7-deazaadenines and hypoxanthines are potent adenosine antagonists.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 31104-20 LBC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

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

P.I.:	D.M. Jerina	Section Chief	LBC, NIDDK
Others:	J. Sayer	Research Chemist	LBC, NIDDK
	H. Yagi	Visiting Scientist	LBC, NIDDK
	S.K. Balani	Guest Worker	LBC, NIDDK
	A. Cheh	Research Chemist	LBC, NIDDK
	D.R. Bushman	Staff Fellow	LBC, NIDDK
	N.T. Nashed	Senior Staff Fellow	LBC, NIDDK
	A. Chadha	Visiting Fellow	LBC, NIDDK

COOPERATING UNITS (if any)

A. Conney and W. Levin, Roche Inst. (Nutley, NJ); D. Whalen, Dept. of Chem., U. of MD. (Catonsville); D. Boyd, Dept. of Chem., Queen's Univ. of Belfast (N. Ireland); D. Thakker, Center for Drugs and Biologics, FDA (Bethesda, MD).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

8.5

PROFESSIONAL:

7.5

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

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

The primary goal has been the elucidation of the structures of reactive metabolites which are responsible for the carcinogenic, cytotoxic and mutagenic activity of polycyclic aromatic hydrocarbons. The approach taken consists of: i) synthesis of primary and secondary oxidative metabolites, ii) study of the metabolism of the hydrocarbons with liver microsomes, as well as with purified and reconstituted cytochrome P-450 systems with and without epoxide hydrolase, iii) tests for mutagenicity of the synthetic metabolites, iv) elucidation of the roles of the cytochrome P-450 system and epoxide hydrolase in potentiating or obliterating the mutagenicity of these metabolites, v) determination of the carcinogenic activity of these compounds, vi) determination of the reaction rates and nature of the products formed by arene oxides and diol epoxides upon reaction with biopolymers and model compounds, and vii) search for agents capable of preventing the tumorigenic action of active metabolites. Current chemical studies have included a new synthesis of oxepins which form during the preparation of benzo-ring arene oxides from dibromoacetate precursors. In selected cases, predictable from PMO calculations, the arene oxides undergo facile photorearrangements to the same oxepins. Studies of the pH dependent solvolysis of precocene I 3,4-oxide have established an unprecedented change in rate determining step from formation of a carbocation intermediate (low pH) to capture of the carbocation by solvent (high pH). Examination of the nucleotide - catalyzed hydrolysis of benzo(a)pyrene 7,8-diol 9,10-epoxide established the importance of stacking interactions between the nucleotide and the hydrocarbon in this general-acid (phosphate) catalyzed process. Stereo-selective metabolism of triphenylene to triphenylene 1,2-oxide enantiomers was shown to be dependent on the cytochrome P-450 preparation utilized. Tumor studies established that introduction of a 6-fluoro substituent into the highly tumorigenic benzo(a)pyrene 7,8-diol 9,10-epoxide eliminates carcinogenic activity, possibly due to a conformational change.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31105-03 LBC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Nicotinic and Muscarinic Acetylcholine Receptor Agonists.

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

P.I.:	J.A. Waters	Research Chemist	LBC, NIDDK
Others:	J.W. Daly	Chief	LBC, NIDDK
	Y. Nishizawa	Guest Worker	LBC, NIDDK

COOPERATING UNITS (if any)

A. Aronstom, Med. Coll., Georgia; T. Gund, Newark Coll. of Eng. & Chem., N.J.; C. Spivak, NIDA, Baltimore, MD; I. Stolerman, U. of London, England.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Potent, semirigid nicotinic acetylcholine receptor agonists have been synthesized in order to better understand the acetylcholine receptor recognition sites. Numerous compounds, generally of the acetyl substituted piperidine and piperazine type, and bicyclic amines of the anatoxin-a type, have been prepared for structure-activity correlations. Computer assisted modeling studies have given minimum energy conformations, superimposability diagrams of the hydrogen bond acceptor and the cationic head onto the template, and electrostatic potentials at the van der Waals surfaces, providing additional information for a rational approach to the design of new, potent agonists. Isoarecolone (1-methyl-4-acetyl-1,2,3,6-tetrahydropyridine) methiodide is the most potent of these synthetic nicotinic agonists as shown in various assays: (i) Torpedo electric tissue (high density of nicotinic receptors), (ii) frog rectus abdominus muscle (neuromuscular receptors), (iii) rat pheochromocytoma PC12 cells (ganglionic receptors), and (iv) rat brain membranes (central receptors). Also, isoarecolone hydrochloride produced nicotine-like discriminative effects in rats. Isoarecolone methiodide is only moderately potent at muscarinic M1 receptors (rat brain) in comparison to acetylcholine and exhibits weak activity at M2 receptors (heart).

Nicotinic agonists and muscarinic agonists/antagonists may be useful in the treatment of cholinergic deficient diseases such as Alzheimer's disease, where reduced levels of acetylcholine, acetylcholine receptors and cholineacetyltransferase are found, and myasthenia gravis, where autoantibodies are directed to the main immunogenic region (MIR) of the alpha-subunit of the nicotinic receptor. A study of the effectiveness of isoarecolone salts in animal models of Alzheimer's disease (systemically and intracerebroventricularly) is in progress.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31106-01 LBC

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mechanistic Enzymology of HIV Proteins, An Approach to Rational Drug Design

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

P.I.:	D.M. Jerina	Section Chief	LBC, NIDDK
Others:	J. Sayer	Research Chemist	LBC, NIDDK
	N.T. Nashed	Senior Staff Fellow	LBC, NIDDK
	J. Baillon	Visiting Fellow	LBC, NIDDK
	L. Pannell	Visiting Scientist	LBC, NIDDK

COOPERATING UNITS (if any)

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Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.5

PROFESSIONAL:

0.4

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

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

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

Methods of enzymology, chemical and enzymatic kinetics, and synthetic and analytical chemistry are being used to develop novel agents targeted against the reverse transcriptase (RT) and protease enzymes of HIV-1. i) Design of a convenient continuous spectral assay for RT is being investigated. ii) Affinity labeling agents and/or mechanism-based inhibitors are being developed to probe the active site of RT. Nucleoside analogs that contain functional groups, including an epoxide and a carbonyl group, that are potentially capable of reacting with nucleophiles at the active site of RT have been prepared and tested for anti-HIV activity in vitro. iii) Available information suggests that the HIV protease is an aspartyl protease. Syntheses of potential inhibitors for this class of protease are underway, and several such compounds have been tested for anti-HIV activity. iv) Three natural products derived from South Pacific marine sponges have been tested against HIV in cell culture. Although none of the compounds tested to date have exhibited anti-HIV activity in vitro, they are potentially useful as enzyme inhibitors and may yield valuable information concerning the active sites of the target enzymes.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31107-01 LBC

PERIOD COVERED

October 1, 1987 to September 30, 1988.

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

Mass Spectrometry of Drugs, Metabolites and Natural Products.

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

P.I.:	D. Jerina	Section Chief	LBC, NIDDK
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	J. Daly	Laboratory Chief	LBC, NIDDK

COOPERATING UNITS (if any)

LC, NHLBI, NIH; Univ. of Canterbury, New Zealand; Hebrew Univ., Jerusalem, Israel; LTD, NHLB, GH, Div. Biochem. Biophys., FDA.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

This project was initiated in LBC to provide specialized mass spectrometry support, primarily but not exclusively with trace organic compounds isolated from biological systems. An essential component for this project consists of support from the staff of the Institutes Section on Instrumentation (LAC, NIDDK). Since inception, three additional mass spectrometers have been obtained. The following new techniques have been added or are under development: liquid chromatography-mass spectrometry, tandem (MS/MS) ion trap mass spectrometry, direct exposure probe mass spectrometry of sensitive compounds, deuterium exchange electron impact-mass spectrometry, ion trap self-chemical ionization mass spectrometry, and routine mass spectra of large biomolecules. Collaboration with LC, NHLBI, NIH results in the use of additional mass spectrometers including a Californian plasma desorption mass spectrometer. A counter-current chromatography mass spectrometry system has been developed. Structures of several biologically active compounds obtained from marine sponges in the South Pacific as well as an alkaloid isolated from the skin of an Australian frog have been elucidated. Samples for analysis now derive from many facilities and researchers outside LBC.

Annual Report of the Laboratory of Molecular Biology

National Institute of Diabetes and Digestive and Kidney Diseases

The Laboratory of Molecular Biology has as its principal goal the understanding of biological processes at the molecular level. The research program involves application of theoretical and experimental methods to a wide variety of problems in molecular genetics, regulation of gene expression in eucaryotes, mechanisms of DNA replication, nucleic acid and protein structure, bioenergetics and transport properties. Among the areas under investigation are the structures and chemical properties of biologically important materials. These include studies of enzyme and immunoglobulin structure by X-ray diffraction, investigations of polynucleotide chemistry, structure and interactions by spectroscopic methods and of proteins and nucleic acids by calorimetry, studies of the organization of DNA and proteins within the eukaryotic nucleus, studies of the effects of supercoiling on biological activity and protein-DNA interaction, as well as theoretical analyses of mechanisms of energy conversion in biological systems, muscle contraction, microtubule formation, ion transport and kinetics. There is an increased interest in more direct studies of biological processes. These investigations include studies of the rearrangements in vivo that lead to the formation of active immunoglobulin genes, of the process of DNA replication in both procaryotes and eucaryotes, of the regulatory proteins that control expression of certain eucaryotic genes, of the effects of macromolecular crowding on function in vivo, of nonheritable antibiotic resistance, and of the mechanism of genetic recombination. Significant contributions have been made in all of these areas during the past year.

Enzyme Structure

The aspartyl proteinase from *Rhizopus chinensis* provides a model for the more physiologically relevant enzymes, renin, pepsin and cathepsin D. We have studied this enzyme in order to understand the mechanism of action. Additional inhibitor studies on the aspartyl proteinase have been carried out. The inhibitors, analogs of pepstatin, provide information regarding the mechanism of action of this class of enzyme and support the concept of rational drug design based on a knowledge of three-dimensional structure.

The structure of the enzyme tryptophan synthase from *Salmonella typhimurium* has been further studied and partially refined. The enzyme, which is bifunctional with both (indole glycerol phosphate to indole) and (indole + serine to tryptophan) reactions, is organized in an approximately linear structure, about 145 Å long. The most striking result is the demonstration of the presence of a tunnel linking the two active sites, thus permitting the diffusion of indole, the product of the reaction, to the active site of the enzyme.

Three-Dimensional Structure of Proteins of the Immune System

The structure of the monoclonal antibody, HyHEL-5 Fab, complexed with its antigen, lysozyme, has been determined in three-dimensions by X-ray

diffraction analysis. This structure has now been almost completely refined in two crystal forms.

The structure of the monoclonal antibody HyHEL-10 Fab has also been determined and almost completely refined. A comparison of these structures together with a third such complex determined elsewhere has enabled us to make some general statements about antibody antigen interactions: 1) The most striking feature is the high degree of complementarity between the antibody and the antigen. 2) It appears that epitopes are mainly made up of several sequentially unrelated segments of polypeptide chain brought into proximity by the folding of the protein. 3) Also it would appear that any part of the protein surface that is accessible to antibody is potentially antigenic.

Studies on the Mechanism of Genetic Recombination

The mechanisms of the transposition-replication reaction of bacteriophage Mu provides a model system for understanding the enzymatic steps involved in various genetic rearrangement reactions. A critical step in the Mu transposition reaction is a pair of DNA strand transfers which generate an intermediate DNA molecule with a branched structure. Efficient formation of this intermediate requires Mu A, Mu B and E. coli HU proteins along with ATP and Mg^{++} . The Mu A protein binds to the Mu end DNA sequences on the donor DNA and makes a pair of single strand cuts to expose the 3' ends of the Mu sequence. This cleaved donor DNA with associated proteins is an active intermediate which completes DNA strand transfer by using a DNA molecule which is bound by Mu B protein as the target. The Mu B protein possesses an ATPase activity which is stimulated by Mu A protein and DNA, and selectively stimulates the utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The proper relative orientation of the two Mu end sequences on the donor molecule is specifically recognized by making use of the energy of DNA supercoiling and a specific geometry of the Mu end DNA segments within the initial synaptic complex. The intermediate DNA molecules can be converted into cointegrates by DNA replication or into simple inserts by nucleolytic cleavages and gap repair. Both of these resolution pathways are supported by an E. coli cell extract and do not require Mu proteins.

Studies of Immunoglobulin Gene Rearrangement

Two new and unusual products of V(D)J recombination in lymphoid cells have been identified. Normal recombination joins the two signal sequences that identify the rearrangement site and, in the complementary reaction, joins the fragments of coding sequences that flank the signals. In extrachromosomal plasmid substrates, these products are found, but there is also a class of "hybrid joints" that link one signal to the coding flank of its partner signal. A second class of "open and shut" events rejoins each signal to its own flank, and is identifiable because of nucleotides lost and/or added at the joint. Both these products supply evidence that DNA strand alignment is not precisely specified in V(D)J recombination.

A specific defect of V(D)J recombination has been identified in lymphoid

cell lines derived from mice with the severe combined immune deficiency (scid) mutation. In extrachromosomal substrates, these cells make signal joints (though with greater imprecision than normal) but are unable to make coding joints. This failure appears sufficient to explain the absence of functional B and T cells in scid mice.

Studies of Functions Involved in Genetic Recombination

We have continued to study complexes of DNA gyrase with defined DNA fragments by low-angle neutron scattering and dynamic light scattering. The particle dimensions obtained from both measurements are much too large for a compact particle with the known molecular weight of the complex. The most plausible model is an oblate ellipsoid with a cavity in its center (somewhat like a doughnut) and with one turn of DNA wrapped around the periphery. This model has attractive features in containing an open space to accommodate the DNA after translocation by the enzyme.

Effects of DNA Supercoiling on the Topological Properties of Nucleosomes

We have asked what effect the presence of nucleosome core particles has on the ability of DNA gyrase to supercoil DNA. Synthetic minichromosomes, constructed by reconstituting complexes of core histones with the closed circular plasmid pBR322, were treated with various amounts of DNA gyrase. We have found earlier that the maximum level of supercoiling that is attainable is nearly identical for protein-free plasmids and for plasmids half-saturated with core histones, even though supercoiling does not result in a loss of histones from the complex. It appears that, at sufficiently high levels of supercoiling, contacts between the core particle and DNA are disrupted in such a way that the DNA bound to histones is no longer constrained. Recent experiments have been addressed to determining the structural perturbation that gives rise to this behavior. By measuring the accessibility to chemical reaction of internal sites at known positions within the folded histone octamer, we have now shown that the nucleosome is not opened up into symmetrical halves under these conditions.

Chromatin Structure and Function

We have continued our studies of chromatin structure in the neighborhood of expressed genes, making use of the globin gene family in chicken erythrocytes as a model system. We investigated further the enhancer which we had found at the 3' end of the adult globin gene. The activity of the enhancer has been analyzed by construction of a series of scanning mutations across the domain, and transfection into primary 9 day embryonic erythrocytes. Of the four previously identified protein-binding regions within the enhancer, only two are important for function in these cells. One of these binds two copies of a novel erythroid-specific factor, which we have named Eryf1. We find that every member of the α and β globin gene families has a site for binding Eryf1 in a neighboring regulatory region. An Eryf1 site is also present in the 3' enhancer of the human γ globin gene. This protein may play a major role in specifying the genes to be activated during erythroid development.

The approximately 37 kD protein has been sufficiently purified to make it possible to obtain an amino acid sequence.

We have also used monoclonal antibodies to identify another erythroid-specific factor, BGPl, which binds to the string of 16 G residues in the promoter, and which appears to be related to, but distinct from, the general factor Spl. We have continued the study of the 3' enhancer, and shown that it can control the embryonic promoter, located 5' of the enhancer, with an activity *in vitro* that partially mimics the stage-specific regulation seen *in vivo*. We have also begun an analysis of the adult globin genes, and shown that their regulatory mechanisms, which share some elements with the family, are nonetheless much simpler. This simplicity is consistent with the limited regulatory repertoire of these genes *in vivo*.

Our results indicate that two kinds of factors are involved in developmental regulation of globin gene expression: Some factors, like Eryfl, are present throughout development and have may be associated with erythroid 'identity'. Others, like BGPl and the palindrome binding factor, affect individual members of the globin family and are accordingly modulated during development.

Developmental Regulation of Differential Gene Expression

The developmental program *in vitro* of differential class III gene expression in *Xenopus laevis* has been reconstructed. A potential molecular basis for oocyte-specific class III gene inactivation and repression has been defined. Oocyte specific class III genes (oocyte 5S RNA genes and satellite I DNA) bind transcription factors with lower affinities than do the somatic type class III genes (somatic 5S RNA genes). Stable transcription complexes are formed on somatic-type class III genes, but not on oocyte-type class III genes. The basis of stability on a somatic 5S RNA gene is the cooperative interaction of two transcription factors (TFIIIA and TFIIIC) with a 10bp region of the promoter. Limitation of transcription factors during embryogenesis, the sequestration of transcription factors or a competing protein-nucleic acid interaction such as chromatin assembly, can all cause the displacement of transcription complexes from oocyte-specific class III genes. Chromatin assembly in the presence of histone H1 leads to both the loss of oocyte-specific class III gene transcription complexes and the establishment of a repressed chromatin structure on this DNA.

Nonheritable Antibiotic Resistance

We have found that salicylate and acetylsalicylate affect the regulation of several outer membrane protein genes in *E. coli*. This results in an altered outer membrane with reduced amounts of OmpF protein. As a consequence, the outer membrane is found to be less permeable to various antibiotics and the salicylate grown cells show increased resistance to diverse antibiotics.

Studies on the mechanism of action of the potent antimicrobial magainins have shown that their activity is strongly concentration-dependent.

Furthermore, they are very sensitive to halide ions: fluoride enormously stimulates while the larger chloride, iodide and bromide ions antagonize their activity.

The human G6PD gene has been inserted in an E. coli plasmid where it can be expressed under the control of a bacterial promoter. The G6PD made in the bacteria can function in place of the bacterial gene demonstrating high conservation of function during evolution.

Influence of Macromolecular Crowding on Biological Systems

The nick-translation reaction of *E. coli* DNA polymerase I (Pol I) was used as a model system to demonstrate the ability of macromolecular crowding to alter the response of an enzyme to a number of basic parameters, such as pH, temperature or inhibitors. In the presence of high concentrations of non-specific polymers, nick translation occurred under a variety of otherwise strongly inhibitory conditions. These crowding effects are accentuated at higher ionic strengths, suggesting their origin in increased binding between the polymerase and its DNA template-primer under crowded conditions. Kinetic measurements were consistent with such a mechanism.

We have also examined the effects of crowding on the association reactions of ribosomal particles. The equilibrium for the binding reaction between the 30 S and 50 S ribosomal subunits of E. coli is shifted towards formation of 70 S ribosomes in the presence of a variety of polymers. The polymers also increase a further interaction between 70 S particles to form the 100 S dimer. The requirement for relatively high concentrations of non-specific polymers indicates that the shifts in equilibria arise from excluded volume effects. Analysis using scaled particle theory is consistent with this mechanism. The effects of high concentrations of polymers on the interactions between ribosomal species may make important changes in the function of ribosomes under the crowded conditions which occur in vivo.

Thermal Measurements of Biomolecular Systems

A four channel differential scanning calorimeter of the heat-flow type has been constructed (with C.P. Mudd), calibrated and placed into operation. The computer software required for data acquisition and analysis has been written (with T.R. Clem) and tested. This instrument will markedly increase experimental throughput.

The thermodynamic basis of the destabilization of some DNA dodecamers caused by the introduction of purine-purine base mismatches has been found to be wholly of entropic origin.

We have demonstrated that when apparent thermodynamic parameters are derived from the temperature dependence of spectroscopic measurements, it is mandatory to carry out calorimetric experiments as well, in order to validate the significance of the spectroscopic data. In the cases we have examined, the direct (and different) values obtained by calorimetry show that meaningful thermodynamic parameters are not obtained by optical methods, and indicate that great caution must be exercised in their application.

Origins of Mammalian DNA Replication in Normal and SV40 Transformed Cells

*Our investigation of the effect of the DNA sequence $(GA)_n'(CT)_n$ on DNA replication has been continued. Synthetic DNA of this sequence, 80 bp in length, was cloned in *E. coli*. When we attempted to insert this sequence into SV40, all of the SV40 isolates grew slowly and had suffered a large deletion of the $(GA)_n'(CT)_n$ sequence, whereas random DNA of the same length gave SV40 variants of the expected size and grew normally. We are currently attempting to use the new blotting procedure of Brewster and Fangman to demonstrate the inhibitory effect of this sequence on DNA replication.

We have continued our investigation of the "O-family" sequence. We have demonstrated the presence of a protein that site-specifically interacts with a portion of the sequence. In collaboration with Dr. Bruce Howard, we have demonstrated that the O-family sequence on transfection into animal cells inhibits DNA synthesis. A small deletion that obliterates the site specific protein interaction, also diminishes the inhibitory effect of the O-family sequence on DNA replication. We also continue to attempt to demonstrate transposon-like activity for the O-family sequences.

Energy Conversion in Biology

A large number of different topics have been studied during the past year in the general field of free energy transduction. The most important areas in which progress has been made are the study of oscillations in microtubule growth dynamics, the role of kinesin in fast axonal transport, free energy transduction by random fluctuations, proton pumping in oxidative phosphorylation, cooperativity in viral fusion, dynamic interactions between electric fields and membrane enzymes, and free energy coupling and the antibiotic action of Magainin-2.

Statistical Thermodynamics of Protein and Polynucleotide Systems

Statistical mechanics have been used to derive the binding of a ligand to a one-dimensional lattice in the presence of another, competing, ligand. The binding isotherm equations derived for the system were then used to analyze the competitive binding data of myosin subfragment-1 and caldesmon to actin.

Chemical and Structural Investigations of Nucleic Acids and Related Macromolecules

We have been investigating chemical and physical properties of self-complementary DNA segments containing specific restriction endonuclease sequences. During the past year we have investigated the basic sequence d-GGTACGCGTACC and the effects of introducing mispairs, two AG and two AI, at different positions in this sequence. One striking result is that when either AG or AI mispairs are introduced at adjacent positions (5,6) in the center of the helix, there is very little change in stability, whereas the same substitutions at positions 5 and 8 cause large reductions in T_m .

In collaboration with Dr. G. Govil of TIFR, Bombay, we have determined by

2D NMR the approximate solution conformation of d-GGTACGCGTACC, which contains recognition sequences for the restriction enzymes RsaI and FnuDII. Complete resonance assignments at 500 MHz have been obtained by 2D correlated spectroscopy (COSY) and nuclear Overhauser enhancement spectroscopy (NOESY). The overall structure is close to B form, though it differs in significant detail from the standard B form of Arnott.

Replication, Recombination and Repair of Microbial DNA

Studies on DNA replication of plasmid ColE1 have been continued. Transcripts (RNA II) that start 555 nucleotides upstream of the replication origin by RNA polymerase form a hybrid with the template DNA. The hybridized transcript is cleaved by ribonuclease H at the origin and used as the primer for leading strand synthesis by DNA polymerase I. ColE1 DNA can replicate also in the absence of the RNase H and DNA polymerase I. RNA II hybridized with the template DNA displaces the nontranscribed strand on which lagging strand synthesis takes place.

RNA II that extends beyond the normal replication origin terminates at various positions. When a stretch of dA residues is inserted at various positions of the template strand downstream of the origin most transcripts that hybridize with the template DNA terminates at the stretch. When RNA II transcription terminates at a dA stretch, a single-stranded region of a defined length is formed on the nontranscribed strand. For initiation of synthesis of the lagging strand, formation of a single-stranded legion of at least 45 nucleotides long is required. The electron microscopic studies show that the host Dna B protein with a helicase activity binds to this short single-stranded region and unwinds the template DNA to allow assembly of a replisome for lagging strand synthesis.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 33000-22 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies of Functions Involved in Genetic Recombination

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

PI: Martin Gellert, Chief, Section on Metabolic Enzymes LMB/NIDDK

Others: James Tamura Guest Worker LMB/NIDDK

Mary H. O'Dea Research Chemist LMB/NIDDK

Hans Westerhoff Guest Worker LMB/NIDDK

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Ms. S. Krueger, University of Maryland, College Park, MD

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

2.50

PROFESSIONAL:

2.50

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Complexes of DNA gyrase with defined DNA fragments were studied by low-angle neutron scattering and dynamic light scattering. The results imply that the structure is an open one, with a solvent-filled cavity at the center of an oblate ellipsoid.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 33001-04 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies of Immunoglobulin Gene Rearrangement

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

PI: Martin Gellert	Chief, Section on Metabolic Enzymes	LMB/NIDDK
Kiyoshi Mizuuchi	Visiting Scientist	LMB/NIDDK
Others: Joanne Hesse	Research Chemist	LMB/NIDDK
Michael Lieber	Guest Worker	LMB/NIDDK
Susanna Lewis	Guest Worker	LMB/NIDDK
David Brown	PRAT Fellow	LMB/NIDDK

COOPERATING UNITS (if any)

Dr. Moshe Sadofsky	LP/NCI
Dr. Melvin Bosma, Fox Chase Center for Cancer Research, Philadelphia, PA	

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

5

PROFESSIONAL:

5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Two new and unusual products of V(D)J recombination in lymphoid cells have been identified. Normal recombination joins the two signal sequences that identify the rearrangement site and, in the complementary reaction, joins the fragments of coding sequences that flank the signals. In extrachromosomal plasmid substrates, these products are found, but there is also a class of "hybrid joints" that link one signal to the coding flank of its partner signal. A second class of "open and shut" events rejoins each signal to its own flank, and is identifiable because of nucleotides lost and/or added at the joint. Both these products supply evidence that DNA strand alignment is not precisely specified in V(D)J recombination.

A specific defect of V(D)J recombination has been identified in lymphoid cell lines derived from mice with the severe combined immune deficiency (scid) mutation. In extrachromosomal substrates, these cells make signal joints (though with greater imprecision than normal) but are unable to make coding joints. This failure appears sufficient to explain the absence of functional B and T cells in scid mice.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Effects of DNA Supercoiling on the Topological Properties of Nucleosomes

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

PI: Martin Gellert	Chief, Section on Metabolic Enzymes	LMB/NIDDK
Gary Felsenfeld	Chief, Section on Physical Chemistry	LMB/NIDDK
Others: David Clark	Visiting Fellow	LMB/NIDDK
Mary H. O'Dea	Research Chemist	LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

.50

PROFESSIONAL:

.50

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

In the nucleosome core particle, at least 145 base pairs of DNA are bound to the histone octamer in a superhelical conformation. We have asked what effect the presence of these particles has on the ability of DNA gyrase to supercoil DNA. Synthetic minichromosomes, constructed by reconstituting complexes of core histones with the closed circular plasmid pBR322, were treated with various amounts of DNA gyrase. We have found that the maximum level of supercoiling that is attainable is nearly identical for protein-free plasmids and for plasmids half-saturated with core histones, even though supercoiling does not result in a loss of histones from the complex. It appears that, at sufficiently high levels of supercoiling, contacts between the core particle and DNA are disrupted in such a way that the DNA bound to histones is no longer constrained. Recent experiments indicate that the nucleosome is not opened up into symmetrical halves under these conditions.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 33006-10 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies on the Mechanism of Genetic Recombination

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

PI: Kiyoshi Mizuuchi, Visiting Scientist LMB/NIDDK

Others: K. Adzuna Visiting Fellow LMB/NIDDK

R. Craigie Visiting Associate LMB/NIDDK

M. Mizuuchi Visiting Fellow LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Genetic Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

3

PROFESSIONAL:

3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The major objective of this project is to uncover the enzymatic steps involved in various genetic rearrangement reactions and to study the mechanism of action of the enzymes involved. We are particularly interested in gene rearrangements caused by transposon family of movable genetic elements. The mechanisms of the transposition-replication reaction of bacteriophage Mu is studied under this project as a model system.

A critical step in the Mu transposition reaction is a pair of DNA strand transfers which generate an intermediate DNA molecule with a branched structure. Efficient formation of this intermediate requires Mu A, Mu B and E. coli HU proteins along with ATP and Mg⁺⁺. The Mu A protein binds to the Mu end DNA sequences on the donor DNA and makes a pair of single strand cuts to expose the 3' ends of the Mu sequence. This cleaved donor DNA with associated proteins is an active intermediate which completes DNA strand transfer by using a DNA molecule which is bound by Mu B protein as the target. The Mu B protein possesses an ATPase activity which is stimulated by Mu A protein and DNA, and selectively stimulates the utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The proper relative orientation of the two Mu end sequences on the donor molecule is specifically recognized by making use of the energy of DNA supercoiling and a specific geometry of the Mu end DNA segments within the initial synaptic complex.

The intermediate DNA molecules can be converted into cointegrates by DNA replication or into simple inserts by nucleolytic cleavages and gap repair. Both of these resolution pathways are supported by an E. coli cell extract and do not require Mu proteins.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 34001-23 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Chromatin Structure and Function

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

PI: Gary Felsenfeld, Chief, Section on Physical Chemistry LMB/NIDDK

OTHERS:

David Clark, Visiting Fellow LMB/NIDDK Joe Knezetic, Guest Researcher LMB/NIDDK
 Stephen Clark, Visiting Fellow LMB/NIDDK Catherine Lewis, Staff Fellow LMB/NIDDK
 Todd Evans, Staff Fellow LMB/NIDDK Mark Minie, Staff Fellow LMB/NIDDK
 Hannah Gould, Expert LMB/NIDDK Joanne Nickol, Research Chemist LMB/NIDDK
 P. David Jackson, Chemist LMB/NIDDK Marc Reitman, Research Chemist LMB/NIDDK
 Takeshi Kimura, Visiting Fellow LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

8.75

PROFESSIONAL:

8.75

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

We have continued our studies of chromatin structure in the neighborhood of expressed genes, making use of the globin gene family in chicken erythrocytes as a model system. We investigated further the enhancer which we had found at the 3' end of the adult β globin gene. The activity of the enhancer has been analyzed by construction of a series of scanning mutations across the domain, and transfection into primary 9 day embryonic erythrocytes. Of the four previously identified protein-binding regions within the enhancer, only two are important for function in these cells. One of these binds two copies of a novel erythroid - specific factor, which we have named Eryf1. We find that every member of the α and β globin gene families has a site for binding Eryf1 in a neighboring regulatory region. An Eryf1 site is also present in the 3' enhancer of the human β globin gene. This protein may play a major role in specifying the genes to be activated during erythroid development. We have also used monoclonal antibodies to identify another erythroid - specific factor, BGp1, which binds to the string of 16 G residues in the β promoter, and which appears to be related to, but distinct from, the general factor Spl. We have continued the study of the 3' β enhancer, and shown that it can control the embryonic ϵ promoter, located 5' of the enhancer, with an activity in vitro that partially mimics the stage-specific regulation seen in vivo. We have also begun an analysis of the adult α globin genes, and shown that their regulatory mechanisms, which share some elements with the β family, are nonetheless much simpler. This simplicity is consistent with the limited regulatory repertoire of these genes in vivo.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Enzyme Structure

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

PI: David R. Davies, Chief, Section on Molecular Structure

LMB/NIDDK

Others: Gerson H. Cohen

Research Chemist

LMB/NIDDK

Craig Hyde

Staff Fellow

LMB/NIDDK

Kaza Suguna

Visiting Fellow

LMB/NIDDK

Eduardo Padlan

Visiting Scientist

LMB/NIDDK

T.N. Bhat

Visiting Scientist

LMB/NIDDK

COOPERATING UNITS (if any)

Edith Miles, LBP, NIDDK

J. Boger, Merck, Sharp & Dohme Research Laboratories .

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.75

PROFESSIONAL

2.75

OTHER

0

CHECK APPROPRIATE BOX(ES)

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

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

1) Additional inhibitor studies on the aspartyl proteinase from *Rhizopus chinensis* have been carried out. The inhibitors, analogs of pepstatin, provide information regarding the mechanism of action of this class of enzyme and support the concept of rational drug design based on a knowledge of three-dimensional structure.

2) The structure of the enzyme tryptophan synthase from *Salmonella typhimurium* has been further studied and partially refined. The enzyme, which is bifunctional with an α and β reaction, is organized in an approximately linear $\alpha\beta\beta\alpha$ structure, about 145 A long. The most striking result is the demonstration of the presence of a tunnel linking the two active sites, thus permitting the diffusion of indole, the product of the α reaction, to the active site of the β enzyme.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 34003-20 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Three-Dimensional Structure of Proteins of the Immune System

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

PI: David R. Davies, Chief, Section on Molecular Structure	LMB/NIDDK
Others: T.N. Bhat Visiting Scientist	LMB/NIDDK
Gerson H. Cohen Research Chemist	LMB/NIDDK
Enid W. Silverton Research Chemist	LMB/NIDDK
Eduardo A. Padlan Visiting Scientist	LMB/NIDDK
Steven Sheriff Senior Staff Fellow	LMB/NIDDK
Christina John Visiting Fellow	LMB/NIDDK

COOPERATING UNITS (if any)

Sandra Smith-Gill, National Cancer Institute, NIH

LAB/BRANCH

Section on Molecular Biology

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

5

PROFESSIONAL

5

OTHER

0

CHECK APPROPRIATE BOXES)

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

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

1) The structure of the monoclonal antibody, HyHEL-5 Fab, complexed with its antigen, lysozyme, has been determined in three-dimensions by X-ray diffraction analysis. This structure has now been almost completely refined in two crystal forms.

2) The structure of the monoclonal antibody HyHEL-10 Fab has also been determined and almost completely refined. A comparison of these structures together with a third such complex determined elsewhere has enabled us to make some general statements about antibody antigen interactions.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 35000-24 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Chemical and Structural Investigations of Nucleic Acids and Related Molecules

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

PI: H. Todd Miles, Chief, Section on Organic Chemistry LMB/NIDDK

Others:	F. B. Howard	Research Chemist	LMB/NIDDK
	J. Frazier	Research Chemist	LMB/NIDDK
	H. Miyashiro	Visiting Fellow	LMB/NIDDK

COOPERATING UNITS (if any)

Girjesh Govil, Tata Institute Fundamental Research, Bombay, India
 Phil Ross, LMB/NIDDK
 Kunal Roy, Jawaharlal Nehru University, New Delhi, India

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Organic Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

4

PROFESSIONAL:

3

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

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

This project has the objective of understanding the chemistry and structure of nucleic acids and relating this knowledge to the biological functions of these molecules. Methods used include chemical synthesis of defined sequence DNA fragments and of enzyme substrates, enzymatic synthesis of polynucleotides, study of nucleic acids by circular dichroism, ultraviolet, infrared, and nuclear magnetic resonance spectroscopy, study of thermal transitions and dependence of physical properties on solution conditions. Subjects of investigation include factors which determine the stability of helical complexes, specificity of nucleic acid interactions, location and affinity of binding sites.

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Replication, Recombination and Repair of Microbial DNA

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

PI:	J. Tomizawa	Chief, Section on Molecular Genetics	LMB/NIDDK
Others:	S. Nakasu	Visiting Fellow	LMB/NIDDK
	M. Brenner	Expert	LMB/NIDDK
	Y. Eguchi	Visiting Fellow	LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Molecular Genetics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Studies on DNA replication of plasmid ColeI have been continued. Transcripts (RNA II) that start 555 nucleotides upstream of the replication origin by RNA polymerase form a hybrid with the template DNA. The hybridized transcript is cleaved by ribonuclease H at the origin and used as the primer for leading strand synthesis by DNA polymerase I. ColeI DNA can replicate also in the absence of the RNase H and DNA polymerase I. RNA II hybridized with the template DNA displaces the nontranscribed strand on which lagging strand synthesis takes place.

RNA II that extends beyond the normal replication origin terminates at various positions. When a stretch of dA residues is inserted at various positions of the template strand downstream of the origin most transcripts that hybridize with the template DNA terminates at the stretch. When RNA II transcription terminates at a dA stretch, a single-stranded region of a defined length is formed on the nontranscribed strand. For initiation of synthesis of the lagging strand, formation of a single-stranded region of at least 45 nucleotides long is required. The electron microscopic studies show that the host Dna B protein with a helicase activity binds to this short single-stranded region and unwinds the template DNA to allow assembly of a replisome for lagging strand synthesis.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36003-04 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Nonheritable Antibiotic Resistance

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

PI:	J. L. Rosner	Research Biologist	LMB/NIDDK
Others:	J. D. Foulds	Research Chemist	LSB/NIDDK
	M. Zasloff	Pediatric Physician	NPM/NICHHD
	M. Aumercier	Visiting Fellow	LMB/NIDDK
Foreign:	Maria Persico, Senior Scientist, Int. Lab. of Gen. & Biophysics, Naples, Italy		

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.25

PROFESSIONAL:

2.25

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Salicylate and acetylsalicylate effect the regulation of several outer membrane protein genes in E. coli. This results in an altered outer membrane with reduced amounts of OmpF protein. As a consequence, the outer membrane is found to be less permeable to various antibiotics and the salicylate grown cells show increased resistance to diverse antibiotics.

Studies on the mechanism of action of the potent antimicrobial magainins have shown that their activity is strongly concentration-dependent. Furthermore, they are very sensitive to halide ions: flouride enormously stimulates while the larger chloride, iodide and bromide ions antagonize their activity.

The human G6PD gene has been inserted in an E. coli plasmid where it can be expressed under the control of a bacterial promoter. The G6PD made in the bacteria can function in place of the bacterial gene demonstrating high conservation of function during evolution.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36051-20 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Origins of Mammalian DNA Replication in Normal and SV40 Transformed Cells

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

PI: Robert G. Martin, Chief, Section on Microbial Genetics LMB/NIDDK

Others: R. L. Lechner Senior Staff Fellow LMB/NIDDK
 B. S. Rao Visiting Fellow LMB/NIDDK
 S. S. Wang Research Chemist LMB/NIDDK

COOPERATING UNITS (if any) Bruce Howard, NCI, NIH

Foreign: M. Zannis-Hadjopoulos, McGill Cancer Center, Montreal, Canada
 G. Kaufmann, Tel Aviv University, Tel Aviv, Israel
 H. Manor, Technicon U., Haifa, Israel

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

3.5

PROFESSIONAL:

3.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Our investigation of the effect of the DNA sequence $(GA)_n \cdot (CT)_n$ on DNA replication has been continued. Synthetic DNA of this sequence, 80 bp in length, was cloned in *E. coli*. When it was attempted to insert this sequence into SV40, all of the SV40 isolates grew slowly and had suffered a large deletion of the $(GA)_n \cdot (CT)_n$ sequence, whereas random DNA of the same length gave SV40 variants of the expected size and grew normally. We are currently attempting to use the new blotting procedure of Brewster and Fangman to demonstrate the inhibitory effect of this sequence on DNA replication.

We have continued our investigation of the "O-family" sequence. We have demonstrated the presence of a protein that site-specifically interacts with a portion of the sequence. In collaboration with Dr. Bruce Howard, we have demonstrated that the O-family sequence on transfection into animal cells inhibits DNA synthesis. A small deletion that obliterates the site specific protein interaction, also diminishes the inhibitory effect of the O-family sequence on DNA replication.

We continue to attempt to demonstrate transposon-like activity for the O-family sequences.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 36101-14 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Energy Conversion in Biology

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

PI: Terrell L. Hill, Chief, Section on Theoretical Molecular Biology, LMB, NIDDK

Others:	Y. Chen, R.C., LMB/NIDDK	R. D. Astumian, S.F., LB/NHLBI
	H.V. Westerhoff, G.W., LMB/NIDDK	R. W. Hendler, Sect. Chief, LCB/NHLBI
	F. Kamp, V.F., LMB/NIDDK	D. Juretic, S.F., LCB/NHLBI
	R.J. Rubin, Expert, LMB/NIDDK	L. J. Rosner, Biologist, LMB/NIDDK
	R. Blumenthal, LMB/NCI	G. Kuipers, V.F., LCBG/NIDDK

COOPERATING UNITS (if any)

K. van Dam, Univ. Amsterdam, Netherlands	M. Zasloff, Univ. Penn., PA
R. Wanders, Univ. Amsterdam, Netherlands	P. Nichols, Brock Univ., Canada
K-J. Hellingwerf, Univ. Gorningen, Netherlands	D. B. Kell, Univ. College Wales, Aberystwyth, UK

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Theoretical Molecular Biology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

4.7

PROFESSIONAL:

4.7

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

A large number of different topics have been studied in the general field of free energy transduction. The most important areas in which progress has been made are the study of oscillations in microtubule growth dynamics, the role of kinesin in fast axonal transport, free energy transduction by random fluctuations, proton pumping in oxidative phosphorylation, cooperativity in viral fusion, dynamic interactions between electric fields and membrane enzymes, and free energy coupling and the Magainin-2 antibiotic action.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36102-17 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Statistical Thermodynamics of Protein and Polynucleotide Systems

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

PI: Terrell L. Hill, Chief, Section on Theoretical Molecular Biology
LMB, NIDDK

Others: Yi-der Chen Research Chemist, LMB, NIDDK

COOPERATING UNITS (if any)

J. Chalovich, Univ. East Carolina, North Carolina

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Theoretical Molecular Biology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.3

PROFESSIONAL

0.3

OTHER

0

CHECK APPROPRIATE BOX(ES)

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

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

Statistical mechanics were used to derive the binding of a ligand to a one-dimensional lattice in the presence of another ligand which compete with the first ligand. The binding isotherm equations derived for the system was then used to analyze the competitive binding data of myosin subfragment-1 and caldesmon to actin.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36104-07 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Thermal Measurements of Biomolecular Systems

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

PI: P.D. Ross Research Chemist LMB/NIDDK

OTHERS:

A.C. Steven	Visiting Scientist	LMB/NIAMS	C.P. Mudd	Engineer	BEI/R
W.A. Hagins	Research Chemist	LCP/NIDDK	T.R. Clem	Engineer	BEI/R
A. Shrake	Research Chemist	DBBP/CDB			
F.B. Howard	Research Chemist	LMB/NIDDK			
H.T. Miles	Research Chemist	LMB/NIDDK			

COOPERATING UNITS (if any)

W. Kirchoff Div. of Chemical Sciences, Dept. of Energy, Germantown, Maryland

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

4

PROFESSIONAL

4

OTHER

0

CHECK APPROPRIATE BOX(ES)

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

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

1. A four channel differential scanning calorimeter of the heat-flow type has been constructed (with C.P. Mudd), calibrated and placed into operation. The computer software required for data acquisition and analysis has been written (with T.R. Clem) and tested. This instrument will markedly increase experimental throughput.

2. The thermodynamic basis of the destabilization of some DNA dodecamers caused by the introduction of purine-purine base mismatches has been found to be wholly of entropic origin.

This investigation (with F.B. Howard and H.T. Miles) has demonstrated that it is mandatory to carry out calorimetric experiments to validate the significance of any apparent thermodynamic parameters that may be derived from the temperature dependence of spectroscopic measurements. The failure to obtain meaningful thermodynamic parameters for these molecules from these optical methods, indicates that great caution must be exercised in their application.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36105-06 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Influences of Macromolecular Crowding on Biochemical Systems

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

PI: S.B. Zimmerman Research Chemist LMB, NIDDK
 Others: S.O. Trach Research Chemist LMB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.7

PROFESSIONAL:

1.0

OTHER

0.7

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type Do not exceed the space provided)

The nick-translation reaction of *E. coli* DNA polymerase I (Pol I) was used as a model system to demonstrate the ability of macromolecular crowding to alter the response of an enzyme to a number of basic parameters, such as pH, temperature or inhibitors. In the presence of high concentrations of non-specific polymers, nick translation occurred under a variety of otherwise strongly inhibitory conditions. The conditions tested included a range of pH values or temperatures or inhibitory concentrations of urea, formamide or ethidium bromide. These crowding effects are accentuated at higher ionic strengths, suggesting their origin in increased binding between the polymerase and its DNA template-primer under crowded conditions. Kinetic measurements were consistent with such a mechanism.

We have examined the effects of crowding on the association reactions of ribosomal particles. The equilibrium for the binding reaction between the 30 S and 50 S ribosomal subunits of *E. coli* is shifted towards formation of 70 S ribosomes in the presence of a variety of polymers. The polymers also increase a further interaction between 70 S particles to form the 100 S dimer. The requirement for relatively high concentrations of non-specific polymers indicates that the shifts in equilibria arise from excluded volume effects. Analysis using scaled particle theory is consistent with this mechanism. The effects of high concentrations of polymers on the interactions between ribosomal species may make important changes in the function of ribosomes under the crowded conditions which occur *in vivo*.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Developmental Regulation of Differential Gene Expression

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

PI: Alan Wolffe

Visiting Associate

LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

.75

PROFESSIONAL

.75

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

I have reconstructed in vitro the developmental program of differential class III gene expression in Xenopus laevis. A potential molecular basis for oocyte-specific class III gene inactivation and repression has been defined. Oocyte specific class III genes (oocyte 5S RNA genes and satellite I DNA) bind transcription factors with lower affinities than do the somatic type class III genes (somatic 5S RNA genes). Stable transcription complexes are formed on somatic-type class III genes, but not on oocyte-type class III genes. The basis of stability on a somatic 5S RNA gene is the cooperative interaction of two transcription factors (TFIIIA and TFIIIC) with a 10bp region of the promoter. Limitation of transcription factors during embryogenesis, the sequestration of transcription factors or a competing protein-nucleic acid interaction such as chromatin assembly, can all cause the displacement of transcription complexes from oocyte-specific class III genes. Chromatin assembly in the presence of histone H1 leads to both the loss of oocyte-specific class III gene transcription complexes and the establishment of a repressed chromatin structure on this DNA.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36108-01 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies on the Mechanism of Retroviral DNA Integration

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

PI: Robert Craigie	Visiting Associate	LMB/NIDDK
Kiyoshi Mizuuchi	Visiting Scientist	LMB/NIDDK
Martin Gellert	Chief, Section on Metabolic Enzymes	LMB/NIDDK
Others: Tamio Fujiwara	Special Volunteer	LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Genetic Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

An essential step in the replication cycle of retroviruses is integration of a DNA copy of the viral genome, made by reverse transcription of viral RNA, into the chromosome of a newly infected cell. The objective of this project is to analyze the molecular mechanism of this integration step and to develop a simple in vitro assay system that may be used to efficiently screen for chemical inhibitors of this step in the viral life cycle. Since there is no known host analogue of this integration reaction, it is hoped that some of these inhibitors will prove to be clinically useful as antiviral drugs against HIV.

We currently use Moloney murine leukemia virus (MoMLV) as a model system for studying the mechanism of retroviral DNA integration. Utilizing a cell-free reaction system, in which extracts of virus-infected cells provide both the viral DNA substrate and protein factors required for integration, we have analyzed the structure reveals that MoMLV integrates by a mechanism that is strikingly similar to the transposition mechanism of a prokaryotic transposon, suggesting that this reaction is highly conserved among many mobile genetic elements.

We have recently developed a cell-free reaction system for integration of exogenously added viral DNA. This system is being exploited to determine which viral polypeptides are required for the integration reaction.

The feasibility of screening for inhibitors of integration by means of the cell-free reaction with endogenous viral DNA has already been demonstrated on a laboratory scale.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36109-01 LMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

AIDS related proteins: Structure and function

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

PI: David R. Davies, Chief, Section on Molecular Structure LMB/NIDDK

OTHERS: Gerson H. Cohen, Research Chemist LMB/NIDDK

Kaza Suguna, Visiting Fellow LMB/NIDDK

C. Craig Hyde, Senior Staff Fellow LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS.

1

PROFESSIONAL

1

OTHER

0

CHECK APPROPRIATE BOX(ES)

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

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

- 1) An investigation of the protease of HIV has been carried out to see whether it has homology with other known proteins.
- 2) The aspartyl proteinase has been examined complexed with several pepstatin like inhibitors.

ANNUAL REPORT OF THE METABOLIC DISEASES BRANCH
National Institute of Diabetes and
Digestive and Kidney Diseases

The general goals of the Branch are to investigate the mechanism of action of hormones controlling ion transport and mineral metabolism and to investigate the immunological and pathological factors mediating kidney disorders. The branch currently includes sections of Mineral Metabolism (Dr. Marx), Endocrine Regulation (Dr. Aurbach) and Kidney Disease (Dr. Balow). Integration of these sections is related to common interests in the pathophysiology of metabolic disorders which interface with the kidney. Systems under study include renal and skeletal tissue, transgenic mice, isolated cells (kidney and parathyroid in culture, hormone receptors (beta adrenergic, parathyroid hormone, calcitonin and 1,25 dihydroxy vitamin D), parathyroid cell growth factors, and T cell and B cell function in disorders of immunoregulation.

Analysis of Hormone Receptor

Interactions of catecholamines with adrenergic receptors and activation of adenylate cyclase are under study with the plasma membranes of several cell systems. Specific receptors have now been identified on turkey erythrocytes, parathyroid cells, pineal cells, rat, guinea pig and monkey lung membrane preparations, rat osteosarcoma cells and rat liver membranes. Control of receptor in isolated cell culture systems is being studied with a view toward gaining knowledge about the molecular biology of receptors and how they are linked to intracellular response systems.

Calcitonin has been shown to decrease intracellular cAMP at concentrations 300-fold lower than those that increase cAMP [Drs. Barsony, Marx].

Receptors for Parathyroid Hormone

Studies in collaboration with Dr. T. Murray (St. Michael's Hospital, Toronto) have led to development of radiolabeled intact bovine parathyroid hormone as a ligand. The radiolabeled agonist binds to receptors in canine renal plasma membranes or in cultured osteosarcoma cells with kinetic properties distinct from those of radiolabeled synthetic amino terminal bioactive fragments.

The radioiodinated parathyroid hormone binds to specific receptors on cultured rat osteosarcoma cells and interaction with these receptors correlates well with stimulation of cyclic AMP production in this cell system.

Primary Hyperparathyroidism and Familial Hypercalcemia

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case findings has produced approximately 85 kindreds for analysis. These studies have allowed segregation of the commonest familial variants into two apparently distinct disease syndromes - familial multiple endocrine neoplasia type 1 (FMEN I) and familial hypocalciuric hypercalcemia (FHH). FHH was distinguished from FMEN I by 1) virtually a 100% penetrance for hypercalcemia before age 20, 2) milder clinical manifestations - low incidence of recurrent nephrolithiasis or recurrent peptic ulceration, 3) no hypercalciuria, 4) normal basal concentrations of gastrin, and 5) poor response to subtotal parathyroidectomy. Distinction between the two syndromes, both inherited as autosomal dominant traits, is important because in FHH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial. FHH accounts for approximately 10% of all unsuccessful parathyroidectomies in hypercalcemia. In FHH the ionized and ultrafiltrable calcium concentration in serum are elevated in proportion to the increase in total calcium. In these patients the filtrable load of calcium is high in association with a marked decrease in renal calcium clearance. Even when these patients become surgically hypoparathyroid, the low renal clearance of calcium is strikingly persistent during calcium infusion. The concentration of parathyroid hormone in plasma is lower in patients with FHH than in typical primary hyperparathyroid patients with similar degrees of hypercalcemia whether assessed by PTH radioimmunoassay or by renal clearance of cAMP or phosphate. The parathyroid glands show hyperplasia in most cases. In several kindreds one or more members have exhibited life-threatening primary hyperparathyroidism in the neonatal period. This may result sometimes from a double dose of the FHH gene. Dispersed parathyroid cells from one severely affected neonate showed a striking decrease in sensitivity of PTH secretion to extracellular calcium. This disorder may reflect mutation in a gene that directs calcium recognition in both the parathyroid and renal tubular cell [Drs. Marx, Streeten, Zimering and Aurbach. MDB; Drs. Spiegel and Weinstein MPB].

Familial multiple endocrine neoplasia type 1 (FMEN I) is an autosomal dominant disorder characterized by hyperfunction of parathyroids, pancreatic islets, and anterior pituitary. Affected organs show features suggestive of increased proliferation. Virtually all subjects expressing the gene show primary hyperparathyroidism. Primary hyperparathyroidism is usually first recognizable between ages 20-40, and it shows a high recurrence rate after subtotal parathyroidectomy (approximately 50% after 10 years). We have evaluated multiple indices for use in screening in a very large kindred. We tested 221 members and newly identified 16 as carriers. Albumin-adjusted calcium and PTH were most useful; gastrin and prolactin analyses

were not useful for screening but showed promise in followup of known carriers. Analysis in this family has revealed linkage to a locus on the long arm of chromosome 11. MEN1 related tumors are being screened for loss of heterozygosity at this locus. Tumors with small deletions could speed identification of the MEN1 gene. [Drs. Marx, S. Bale, A. Bale, Mulvihill, Sparkes, Brandi, Aurbach, Sakaguchi].

With cultured bovine parathyroid cells, we found abnormally high mitogenic activity in plasma from 23 of 27 subjects with FMEN1. Well-characterized growth factors or known parathyroid secretagogues showed far less parathyroid mitogenic activity than these FMEN1 plasmas. The mitogenic factor(s) appear to be a protein of 14,000 mw. We have begun purifying this factor for further characterization. We have obtained evidence that the factor is related to basic fibroblast growth factor. Analysis of plasmas from one large kindred with FMEN1 suggests that high parathyroid mitogenic activity precedes primary hyperparathyroidism and may begin at very early ages. [Drs. Brandi, Sakaguchi, Aurbach, Goldsmith, Zimring, Marx].

Studies on noninvasive and invasive modes of localizing parathyroid tumors continue. Parathyroid adenoma localization has been evaluated using the new non-invasive magnetic resonance imaging technique. Initial results were disappointing but the acquisition of a specialized neck collar has led to better resolution in the paratracheal and mediastinal areas. Patients are currently under evaluation with this new technique. A high degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded, in approximately 450 of cases tested, the identification of abnormal masses of tissue proven at surgery to be parathyroid. In the most difficult cases, localization of parathyroid tissue can be aided by identifying high concentrations of parathyroid hormone by radioimmunoassay in veins draining the lesion. Fine needle aspiration is another new method that can obviate other invasive localization procedures. We have aspirated with guidance by computerized tomography or ultrasound approximately 20 such lesions that were subsequently confirmed surgically as parathyroid. RIA of the aspirates showed high concentrations of PTH in all but one. Eight mediastinal adenomas have been treated nonsurgically by percutaneous injection via catheter of occlusive agents into the arterial blood supply with 7 complete and one partial remissions. [Drs. Aurbach, Marx, Spiegel, Nanes, Zimring, Weinstein, Streeten, NIDDK; Dr. Norton, NCI, Drs. Doppman, Miller, and others, Diagnostic Radiology, CC].

Rapid determination of intraoperative UcAMP excretion (using the Gammaflo machine for rapid cAMP radioimmunoassay) has proven to be a valuable tool in guiding surgery for primary hyperparathyroidism, particularly in patients with multigland disease. Persistent elevation of UcAMP requires continued search for abnormal tissue even after 1 or more abnormal glands have

been removed. A rapid (mean 1.5 hours) drop in UcAMP to less than 50% of the baseline range obviates the need for continued exploration even in cases where histologic confirmation of parathyroidectomy is lacking. Spurts in UcAMP above baseline may provide a clue to the location of abnormal parathyroid tissue. [Drs. Spiegel, Marx, Nanes, Zimring, Weinstein, Streeten, and Aurbach, NIDDK: Dr. Norton, NCI Surgery].

Determination of urinary cAMP excretion postoperatively in patients undergoing neck exploration for primary hyperparathyroidism is a useful method for assessing postoperative parathyroid function. UcAMP excretion declines postoperatively in all patients in whom hypercalcemia is corrected but not in those with persistent hypercalcemia. In patients becoming severely hypocalcemic (and requiring vitamin D therapy) postoperatively, UcAMP measurement enables one to distinguish patients with decreased parathyroid reserve as the cause for hypocalcemia (low UcAMP excretion) from patients with healing osteitis fibrosa ("hungry bones") with secondary hyperparathyroidism as the basis for hypercalcemia. UcAMP in the latter group is often elevated but can be suppressed if serum calcium is normalized. Elevated UcAMP excretion postoperatively in the face of hypocalcemia enables one to predict that vitamin D therapy will be required temporarily (if at all) and precludes the need for parathyroid autografts. [Drs. Spiegel, Marx, Zimring, Weinstein, Streeten, and Aurbach, NIDDK].

Postoperative patients with surgically corrected hyperparathyroidism are being actively evaluated in a five year follow up study [Dr. Udelsman, Norton NCI, Drs. Marx, NIDDK]. These patients are being studied for sequelae such as hypoparathyroidism, recurrent hyperparathyroidism, and complications such as vocal cord paralysis.

Secretion of Parathyroid Hormone

PTH secretion from parathyroid glands in vivo and cells in vitro is controlled by intracellular calcium and cyclic AMP. Control by calcium is altered in certain pathologic states (glandular adenomas, carcinomas and perhaps hyperplasia). Agents that alter cellular cAMP change PTH secretion in the same direction. Calcium decreases cellular cAMP, but most of its effect to inhibit secretion is independent of changes in cellular cAMP.

Calcium inhibition of parathyroid hormone secretion was evaluated utilizing pertussis toxin as a probe. Pertussis toxin catalyzes ADP-ribosylation and inactivation of inhibitory guanine nucleotide regulatory proteins, N_i s. Studies in dispersed bovine parathyroid cells indicates that calcium inhibition of parathyroid hormone secretion is mediated via an N_i . Further studies with calcium channel agents show that calcium channels are involved in regulation of PTH secretion.

KIDNEY DISEASES

The research activities of the Kidney Disease Section are focussed on the pathogenesis of immunologically mediated glomerular diseases, including the proliferative and membranous forms of lupus nephritis and of membranoproliferative glomerulonephritis. These diseases are intensively studied to define the immunopathogenesis of the renal lesions in murine and human nephropathies, as well as to delineate the disease modifying effects of several immunosuppressive drug therapies.

I. Glomerulonephritis

A. Immunopathogenesis. Murine models are being utilized to investigate the different forms and components of lupus nephritis. The characteristics of the renal immune complex deposits and the lymphoid cell infiltration are being dissected by immunohistologic and electron microscopic techniques. The effects of various immunomodulating drugs on immunologic features and on the renal lesions are being investigated. Studies of differences among the murine strains have provided new approaches to study of the diverse manifestations and response to treatment of human lupus nephritis. (Austin, Cadena, Balow).

B. Immunoregulatory studies. A multiplicity of T and B lymphocyte abnormalities have been found in patients with systemic lupus erythematosus (SLE). Heightened and poorly regulated B cell activity is characteristic of SLE. Defective T suppressor cell activity is not a consistent finding in all cases of active SLE. Moreover, T cytotoxic cell and natural killer cell activities are deficient and could permit the emergence of abnormal and unregulated autoantibody producing cells. An alternative immunoregulatory defect leading to excessive B cell activity has been noted in certain lupus mouse strains, namely, T helper cell hyperactivity. Our group has found increased numbers of circulating T cells bearing activation markers and proto-oncogene expression which may lead to increased immunoglobulin secretion by autologous B cells. Studies are in progress to ascertain different mechanisms which underlie the heightened production of pathogenic antibodies by B cells in different subsets of patients with lupus nephritis. (Tsokos, Eleftheriades, Boumpas, Patel, Balow).

C. Proliferative lupus nephritis. Current protocols are designed to increase and refine the therapeutic index of different immunosuppressive drugs for lupus nephritis. Studies to date have shown that cytotoxic drugs are superior to conventional corticosteroid therapy and that intermittent high-dose therapy maintains efficacy while reducing toxicity. Patients with proliferative forms of lupus nephritis are being intensively treated with pulse methylprednisolone or pulse cyclophosphamide to compare these two types of drugs and also to assess whether intensity or duration of cyclophosphamide therapy is more important in stabilizing the renal disease. Laboratory studies

of lymphoid cell modulation by the various drug regimens are ongoing in order to maximize efficacy and to improve monitoring. (Balow, Austin, Webb and members of ARB, NIAMS).

D. Membranous lupus nephropathy. This form of lupus nephritis produces substantial morbidity from nephrotic syndrome and causes an insidious loss of renal function. Preliminary evidence indicates that the immunopathogenesis of membranous nephropathy is distinct from that of proliferative lupus nephritis. Ongoing studies include examination of the pathophysiology and histopathology of membranous lupus nephropathy and evaluation of the comparative efficacy of corticosteroids, cyclophosphamide and cyclosporin A on this disease. (Balow, Austin, Webb).

II. Role of Complement in Glomerulonephritis.

A. Nephritic factors. Patients with membranoproliferative glomerulonephritis and lupus nephritis develop autoantibodies to complement converting enzymes associated with abnormal consumption of complement components. These so called nephritic factors may participate in the pathogenesis of the renal diseases, but studies of their exact role has been hindered by insufficient quantities of homogeneous materials. Epstein-Barr virus transformed and sustained B cell lines which actively produce nephritic factors have been produced. One line from a patient with membranoproliferative glomerulonephritis secretes an IgG antibody which binds and stabilizes the alternative pathway C3 convertase enzyme. Another from a patient with lupus binds the classic pathway C3 convertase. Nephritic factors with these functional activities correspond to known abnormalities of complement activation through the different pathways in these disease. The characteristics of the ligand binding sites, turnover and modulation of these autoantibodies are under study. (Tsokos, Thyphronitis, Balow).

B. Complement in immune regulation. Abnormal levels of complement components and deposition in sites of immunological reactions are characteristic of several forms of nephritis. The interactions of complement components and activation products with receptors on lymphoid cells are being studied to gain new insights into their potential role in lupus nephritis, membranoproliferative glomerulonephritis and other renal disorders. The precise role of complement receptors on B cell functions may be particularly relevant to the appearance of autoantibodies associated with these diseases. Studies are underway to determine the mechanism of the modulation of B cell responses through interaction of the complement receptor with natural complement ligands, Epstein-Barr virus and monoclonal antibodies. (Tsokos, Thyphronitis, Pillemer, Balow).

Studies of the pathogenesis of glomerulosclerosis

This laboratory has been interested in the cellular mechanisms leading to glomerular scarring. The hypothesis is that abnormalities in the growth regulation of resident glomerular cells play a major role in the development of glomerulosclerosis. This is being studied using both *in vivo* and *in vitro* approaches. The work focuses on the effect of growth factors and oncogenes on glomerular cells *in vitro*, and on the renal lesions in transgenic mice. (L. Striker, G. Striker, T. Doi, K. MacKay, F. Conti, S. Elliot and L. Agodoa)

III. Glomerulosclerosis (L. Striker, G. Striker)

A. Transgenic Mice SV 40. We have described the renal lesions occurring in mice transgenic for the early region of simian virus 40 that develop proteinuria and a progressive glomerulosclerosis. We have postulated that the glomerular lesions are caused by the presence of T antigen which causes an abnormal cell proliferation. We are now investigating whether a unilateral nephrectomy increases the expression of this antigen, and accelerates the renal lesion. (K. MacKay, L. Striker, G. Striker)

We have shown that mice transgenic for growth hormone and growth hormone-releasing factor which overproduce these hormones develop severe glomerulosclerosis. By contrast mice transgenic for IgF-I develop large glomeruli which remain normal. The glomerular lesions in the GH and GHRF mice mimic those observed in human diabetes mellitus. These models provide a new tool to understand the role of growth hormones in the pathogenesis of glomerulosclerosis. (T. Doi, L. Striker, G. Striker)

B. Studies of Glomerular Cells in Vitro.

1. Murine cells: We have developed lines of mouse glomerular cells both from SV 40 transgenic mice and from their normal littermates. We are now investigating their response to growth stimuli.

2. Insulin and IGF-I: Mouse mesangial cells express surface receptors for IGF-I but not for insulin. IGF-I is mitogen for these cells *in vitro*. (F. Conti, K. MacKay, S. Elliot)

In addition these cells produce and release an IGF-I molecule, which suggests that this hormone as an important autocrine function. (F. Conti, S. Elliot)

3. Endothelial cells and insulin: We have identified the presence of a receptor for insulin in the surface of a clone of endothelial cells from normal mice. (S. Elliot, F. Conti)

4. Transforming growth factors Beta: We have explored the potential regulatory effects of TGF-B on murine glomerular cells. Mouse epithelial, mesangial and endothelial cells as well as isolated rat glomeruli possess high affinity receptors for TGF-B. While TGF-B inhibits the proliferation of epithelial and endothelial cells, it acts as a bifunctional regulator of mesangial cell proliferation. TGF-B increases the biosynthesis of fibronectin and collagen by mesangial cells. These data suggest that TGF-B may play an important role *in vivo* in diseases characterized by resident cell proliferation and accumulation of extracellular matrix. (MacKay)

C. Human Cells.

1. Mesangial cells: We have shown that human mesangial cells possess a receptor for IgF-I but not for insulin. IgF-I constitutes a progression factor for these cells, when these are made competent by PDGF. (T. Doi, S. Elliot)

2. Epithelial cells: Epithelial cells previously infected by a recombinant ADS SV 40 hybrid virus have been propagated and their surface molecules studied using monoclonal antibodies developed in G. Andres' laboratory. We have identified a surface molecule which appears to be unique to glomerular epithelial cells. (T. Doi, S. Elliot)

IV. Kidney Disease in Diabetes

A. Kidney Disease in the Pima Indians. We have undertaken a retrospective study of the glomerular lesions occurring in the Pima Indians who have a very high frequency of NIDDM. We are developing morphometric methods to measure the glomerular surfaces and determine whether glomerulosclerosis is associated with hypertrophy. (L. Striker, L. Agodoa, P. Bennett, G. Striker)

B. Kidney Disease in NOD Mice. We are studying in association with M. Hattori (Joslin Institute, Boston, MA) the glomerular lesions occurring in NOD diabetic mice. We have shown that these mice, whether or not diabetic, are highly susceptible to glomerulosclerosis. The occurrence of diabetes however leads to a striking acceleration of mesangial sclerosis together with a marked proteinuria. The lesions are currently being analyzed. (T. Doi, L. Agodoa)

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43002-23 MD

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Structure, Secretion and Mechanism of Action of Parathyroid Hormone

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

PI: G.D. Aurbach, M.D.

Chief, MDB, NIDDK

OTHERS: L.A. Fitzpatrick, M.D.

Former Senior Staff Fellow, MDB, NIDDK

M.L. Brandi, M.D.

Visiting Associate, MDB, NIDDK

K. Sakaguchi, M.D.

Visiting Fellow, MDB, NIDDK

M. Zimering, M.D.

Medical Staff Fellow, MDB, NIDDK

A. Falchetti, M.D.

Guest Worker, MDB, NIDDK

A. Fattorossi

Guest Worker, MDB, NIDDK

COOPERATING UNITS (if any)

Laboratory of Biochemical Genetics, NHLI

Endocrine Unit, Massachusetts General Hospital

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Endocrine Regulation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

4

PROFESSIONAL:

2.25

OTHER:

1.75

CHECK APPROPRIATE BOXES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided)

It is the purpose of this project to study the secretion, function, and mechanism of action of parathyroid hormone, its relationship to human disease, and to develop clinically useful tests for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine, porcine, rat and human parathyroid hormone have been determined. Synthetic polypeptides representing bovine rat and human parathyroid hormone have been synthesized. These molecules show all the biological properties of the native hormonal polypeptides. Highly sensitive radioimmunoassays for the hormone have been developed and are being modified further for improved clinical diagnostic parameters. Studies show that the mechanism of action of the hormone is mediated through direct hormonal activation of adenylate cyclase in bone and kidney. Isolated parathyroid cells and culture systems have been developed that allow studies on secretory control of parathyroid hormone, and provide test systems to elucidate the pathophysiology of certain hypoparathyroid and hyperparathyroid. states.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43003-23 MD

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies on the Mode of Action of Thyrocalcitonin

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

PI: S.J. Marx, M.D. Chief, Min. Metab. Sec. MDB, NIDDK

Others: J. Barsony, M.D. Guest Researcher MDB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Mineral Metabolism Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

0.1

PROFESSIONAL:

0.1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The purpose is to study the interaction of calcitonin with its specific receptor target organs. The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful for characterizing hormone receptors in kidney, bone and other tissues. Studies are in progress to characterize further the interaction of calcitonin with tissue receptors. It will also be of interest to solubilize the receptors and characterize them chemically. Calcitonin increases cAMP in MCF 7 breast cancer cells. At 300-fold lower concentration calcitonin decreases cAMP in these cells. The decrease in cAMP is prevented by preexposure of cells to agents that interfere with inhibitory guanyl regulatory proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43004-23 MD

PERIOD COVERED

October 1, 1987 through February 28, 1988

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

Studies on pseudohypoparathyroidism and related disorders

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

PI: A. Spiegel, M.D.

Chief, Molecular Pathophysiology
Section

MDB

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Molecular Pathophysiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project transferred to the Molecular Pathophysiology Branch effective February 28, 1988. The new project number is Z01 DK 59002-23 MPB.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43005-23 MD

PERIOD COVERED

October 1, 1987 to February 28, 1988

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

Guanine nucleotide binding proteins as receptor-effector couplers

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

PI: A. Spiegel, M.D.

Chief, Molecular Pathophysiology
Section

MDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Molecular Pathophysiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project transferred to the Molecular Pathophysiology Branch effective February 28, 1988. The new project number is Z01 DK 59001-23 MPB.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43006-13 MD

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Study of Hyperparathyroidism: Etiology, Diagnosis and Treatment

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

PI: G.D. Aurbach, M.D. Chief, MDB, NIDDK

OTHERS: S.J. Marx, M.D.	Ch., Min. Met. Sec., MDB, NIDDK
A.S. Spiegel, M.D.	Ch., Molec. Patho. Sec., MDB, NIDDK
M.S. Nanes, M.D., Ph.D.	Medical Staff Fellow, MDB, NIDDK
L. Weinstein, M.D.	Medical Staff Fellow, MDB, NIDDK
M. Zimering, M.D.	Medical Staff Fellow, MDB, NIDDK
L. Streeten, M.D.	Medical Staff Fellow, MDB, NIDDK

COOPERATING UNITS (if any)

Radiology Department, CC; Surgery Branch, NCI; Digestive Diseases Branch, NIDDK

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Endocrine Regulation Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4.75

PROFESSIONAL:

2.50

OTHER:

2.25

CHECK APPROPRIATE BOX(ES)

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

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

The project goal is the evaluation and treatment of hyperparathyroidism. Patients with persistent or recurrent hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the multiple endocrine neoplasia syndromes. Evaluation ranges from epidemiologic studies of families to in-house studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include radioimmunoassay of parathyroid hormone, ultrasonography, radiothallium scanning, magnetic resonance imaging, CAT scanning, selective arteriography and selective venous sampling for parathyroid hormone, parathyroid gland cryopreservation and autotransplantation, and transcatheter parathyroid gland infarction.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43008-07 MD

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Vitamin D Resistance and Related Disorders

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

PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec.	MDB, NIDDK
Others:	M. Nanes, M.D., Ph.D.	Medical Staff Fellow	MDB, NIDDK
	J. Barsony, M.D.	Guest Researcher	MDB, NIDDK
	M.L. Brandi, M.D.	Visiting Associate	MDB, NIDDK
	G.D. Aurbach, M.D.	Chief	MDB, NIDDK
	W. McCoy	Chemist	MDB, NIDDK

COOPERATING UNITS (if any)

Metabolism Unit, Beilinson Hospital, Petah Tiva, Israel
 Biochemistry Department, University of Arizona, Tucson
 Biochemistry Department, University of Wisconsin, Madison

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Mineral Metabolism Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

2.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The calciferols were the first class of hormonally active steroids to be discovered and also the first for which subjects with hormone resistance could be identified. With recognition that vitamin D is the precursor for 1,25-dihydroxyvitamin D, it has become possible to characterize defects in the activation (1-hydroxylation) of vitamin D and defects in the target action of activated (1,25-dihydroxy)vitamin D. We have demonstrated a broad spectrum of manifestations of hereditary resistance to 1,25(OH)2D ranging from infantile rickets with alopecia and no intestinal response to calciferols to adult onset osteomalacia with satisfactory intestinal response to high doses of calciferols and with no epidermal abnormalities. Alopecia is found only in cases with the most severe grades of resistance to 1,25(OH)2D. This finding implicates the 1,25(OH)2D receptor, for the first time, in normal function of a tissue (hair follicle) outside the classical target in duodenal mucosa. A similar disorder has been recognized in new world monkeys. Cultured skin fibroblasts display many components of the 1,25(OH)2D effector system. Skin fibroblasts from all subjects with hereditary resistance to 1,25(OH)2D display abnormalities in this effector system, and defects in many discrete steps of this pathway have been identified with these cells. Other cells, such as bone cells, lymphocytes, and parathyroid cells can also be used to evaluate actions of 1,25(OH)2D in vitro. Cells with mutations in the 1,25(OH)2D effector pathway can be used to explore mechanisms of calciferol action. They have been used to establish that the 1,25(OH)2D receptor mediates an extremely rapid (1-3 minutes) rise of cyclic GMP in response to 1,25(OH)2D3.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
 NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
 Z01 DK 43009-03 MD

PERIOD COVERED
 October 1, 1987 to September 30, 1988

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

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)
 PI: S.J. Marx, M.D. Chief, Min. Metab. Sec. MDB, NIDDK

Others:	M. Nanes, M.D., Ph.D.	Medical Staff Fellow	MDB, NIDDK
	M.L. Brandi, M.D.	Visiting Associate	MDB, NIDDK
	W. McCoy	Chemist	MDB, NIDDK
	G. Aurbach, M.D.	Chief	MDB, NIDDK
	E. Streeten, M.D.	Medical Staff Fellow	MDB, NIDDK
	K. Sakaguchi, M.D.	Visiting Fellow	MDB, NIDDK

COOPERATING UNITS (if any)
 EEB, CEB, LB, NCI
 Belvedere Medical Center - Carlisle, PA.

LAB/BRANCH
 Metabolic Diseases Branch

SECTION
 Mineral Metabolism Section

INSTITUTE AND LOCATION
 NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.5	OTHER: 0.5
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CHECK APPROPRIATE BOX(ES)

<input checked="" type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input checked="" type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)
 Disorders of mineral metabolism have been evaluated with methods extending from epidemiology to cellular biology. Two forms of familial hyperparathyroidism have been characterized in detail. Familial hypocalciuric hypercalcemia is an autosomal dominant trait associated with abnormal interactions with calcium in parathyroid and kidney. Familial multiple endocrine neoplasia type 1 is an autosomal dominant trait causing hyperfunction of parathyroids, pancreatic islet and anterior pituitary. It is associated with gradual but abnormal proliferation of the tissues affected. Genetic linkage studies in a large kindred have localized the MEN1 gene to the long arm of chromosome 11. Plasma from affected persons shows high mitogenic activity upon cultured bovine parathyroid cells. This mitogenic activity in plasma may be detectable early in life and may be the cause of primary hyperparathyroidism in familial multiple endocrine neoplasia type 1.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43200-09 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Disorders of Immune Regulation in Patients with Systemic Lupus

Erythematosus

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

P. I.: G. C. Tsokos, Guest Researcher, MDB, NIDDK
 Others: J. E. Balow, Senior Investigator, MDB, NIDDK
 D. T. Boumpas, Visiting Associate, MDB, NIDDK

COOPERATING UNITS (if any)

Clinical Center (Aneeta Patel, Biologist).

Foreign: None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

2.00

PROFESSIONAL:

1.75

OTHER:

.25

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

Patients with systemic lupus erythematosus have been found to have various disturbances of the cell-mediated immune response. Cellular aberrations include enhanced spontaneous B cell activity with abnormal triggering in vitro, deficient immunoregulatory T cell circuits, deficient cytotoxic responses, including natural killer cell activity, alloantigen and viral cytotoxicity, and abnormal production of and response to different lymphokines as well as increased expression of proto-oncogenes in highly activated peripheral blood lymphocytes. The goal of these studies is to elucidate further the mechanisms of these alterations of the immune system which are apparently involved in the pathogenesis of this disease. The modulation of the above disturbances by immunosuppressive agents, i.e. corticosteroids and cyclophosphamide, is actively studied, aiming at the restoration of normal immune status in these patients.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43201-04 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Production and Characterization of Nephritic Factors

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

P. I.: G. C. Tsokos, Guest Researcher, MDB, NIDDK
 Others: G. Thyphronitis, Visiting Fellow, MDB, NIDDK
 J. E. Balow, Senior Investigator, MDB, NIDDK

COOPERATING UNITS (if any)

Roger Spitzer MD, Professor of Pediatrics, SUNY, Syracuse NY

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

0.75

PROFESSIONAL:

0.75

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The nephritic factor of the alternative pathway of complement (NeFa) has been found in the sera of patients with membranoproliferative glomerulonephritis (MPGN) and partial lipodystroph (PLD) and has been described as a factor which is able to include cleavage of the third complement (C3) in normal human serum through the alternative pathway. It has been demonstrated that NeFa binds to and stabilizes C3bBb (alternative C3 convertase). NeFa appears to be antigenically and structurally similar to IgG and therefore it might be an autoantibody directed against C3bBb complex. Sera from patients with systemic lupus erythematosus (SLE) contain autoantibodies which bind and stabilize the C3 convertase of the classical pathway. This is classical pathway nephritic factor (NeFc). The relation between the development of renal lesions and the NeFa mediated persistent hypocomplementemia remains unexplained. To study the production of nephritic factors we isolated B lymphocytes from peripheral blood mononuclear cells from patients with MPGN, SLE and normal individuals and established B cell lines by infecting them with Epstein-Barr virus (EBV) containing supernatants. We found that EBV transformed B cell lines derived from patients with MPGN, but not from normal individuals, produce an IgG molecule which stabilizes that C3bBb convertase activity. Supernatants from EBV transformed B cell lines from patients with SLE contain IgG molecules which stabilize C4b2a convertase activity. Full chemical and functional characterization of these antibodies to convertases is in progress.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43202-05 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Regulation of Human Immune Response by Complement

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

P. I.: G. C. Tsokos, Guest Researcher, MDB, NIDDK
 Others: G. Thyphronitis, Visiting Fellow, MDB, NIDDK
 S. R. Pillemer, Medical Staff Fellow, MDB, NIDDK
 J. E. Balow, Senior Investigator, MDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Complement factors and breakdown products acting through cell surface membrane receptors block the differentiation of human B lymphocytes into immunoglobulin secreting cells. Complement receptors are associated with B cell surface immunoglobulin under certain circumstances. Furthermore, complement receptor expression is cell cycle dependent and increased among the cells activity secreting immunoglobulin. Understanding of the mechanism of regulation of immune responses by complement and the role of complement receptors on human B cells is crucial for the understanding of the immunopathogenesis of autoimmune diseases since they are frequently associated with complement activation, depression of complement factor levels and changes in complement receptors.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43204-08 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Immunosuppressive Drug Therapy in Lupus Glomerulonephritis

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

P. I.: J. E. Balow, Senior Investigator, MDB, NIDDK

Others: H. A. Austin, Expert, MDB, NIDDK

COOPERATING UNITS (if any)

NIAMS (Drs. J. Klippel, P. Plotz, A. Steinberg, R. Wilder).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.2

PROFESSIONAL:

1.4

OTHER:

0.8

CHECK APPROPRIATE BOX(ES)

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

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

The efficacy of intensive, intermittent immunosuppressive drug therapy will be evaluated in patients with active lupus glomerulonephritis over a 30 month study period. Patients with renal biopsy documented active glomerulonephritis with or without renal functional deterioration will be treated with low dose corticosteroids and randomized to receive (a) intravenous pulse methylprednisolone monthly for 6 months or (b) intravenous pulse cyclophosphamide monthly for 6 months or (c) intravenous pulse cyclophosphamide monthly for 6 months and then every 3 months for the remaining 24 months of the study. During the final 24 months of the study, all patients will receive low dose prednisone. Active disease, as manifested by renal functional deterioration, increased proteinuria or worsened urinary sediment, will be treated by increased prednisone. Comparison will be made of the number of favorable outcomes of renal function, glomerular pathology and drug related toxicities achieved by each treatment group at the end of the 6th and 30th study months. Between April 1981 and July 1987 there have been more than 60 patients entered into this protocol.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43205-11 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Renal Biopsy Pathology in Systemic Lupus Erythematosus

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

P. I.: J. E. Balow, Senior Investigator, MDB, NIDDK

Others: H. A. Austin, Expert, MDB, NIDDK

COOPERATING UNITS (if any)

Clinical Center: (Dr. D. E. Webb)
Armed Forces Institute of Pathology, Washington, D. C.

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

.25

PROFESSIONAL:

.25

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

The pathogenetic mechanisms underlying the different forms of lupus nephritis are being investigated. Detailed analysis of renal biopsy pathology is being conducted on specimens from patients with systemic lupus erythematosus. Biopsies are classified by major category of lupus nephritis, as well as scored on a semiquantitative scale for specific histologic changes indicating the degree of activity and of chronic sclerosing features. The patterns of immune complex deposition and lymphoid cell interaction with different segments of the nephron are being investigated by immunohistologic techniques and electron microscopy. Morphometric techniques are being developed to analyze more precisely the changes in renal pathology during the course of lupus nephritis. These approaches have facilitated the analysis of the effects of various types of immunosuppressive agents used to halt the progression of lupus nephritis and they will enhance our understanding of the pathogenesis of this disease.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43210-04 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Glomerular Disease in Transgenic Mice

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

PI: K. MacKay	Medical Staff Fellow	MDB, NIDDK
Others: G. Striker	Director	DKUHD, NIDDK
L. Striker	Expert	MDB, NIDDK
S. Elliot	Biologist	MDB, NIDDK
L. Agodoa	Medical Officer	MDB, NIDDK

COOPERATING UNITS (if any)

School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania (R. Brinster).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Mice transgenic for SV40 develop progressive glomerulosclerosis. We have postulated that the disease is due to an abnormal proliferative stimulus (T antigen) leading to mesangial proliferation. We are now investigating whether a stimulus known to induce renal hypertrophy results in an acceleration of the glomerular lesions. To that effect we have performed unilateral nephrectomies in SV40 6 weeks old mice and are examining the expression of T antigen and growth factors (IGF-I, TGF-B).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43211-04 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Histopathology of Renal Lesions in Pima Indians

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

PI:	L. Striker	Expert	MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	F. Conti	Visiting Fellow	MDB, NIDDK
	L. Agodoa	Medical Officer	MDB, NIDDK

COOPERATING UNITS (if any) Epidemiology and Clinical Research Branch, NIDDK, Phoenix, Arizona (P. Bennett). Director of Nephrology at Stanford University, Stanford, California (B. Myers). Howard University, Washington, D.C. (B. Brenner) and Emory University, Atlanta, Georgia (W. Mitch).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

.5

PROFESSIONAL:

.5

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Autopsies from diabetic and non-diabetic Pima Indians will be examined from a series drawn as a representative sample of the autopsy population by Dr. Peter Bennett. Routine light microscopic studies, and potentially electron microscopic studies, will be performed to assess the histopathologic lesions present in these autopsy specimens. Particular attention will be paid to epithelial basement membranes and vascular extracellular matrix areas.

In addition, the PI is chairman of the Natural History Committee of the Pima Indian Project funded by contracts N01-DK-7-2291 and N01-DK-6-2285. The Natural History portion will correlate the histologic, renal physiologic and cell biologic aspects of the kidney disease of diabetes mellitus of the Pima Indians.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43212-04 MD

PERIOD COVERED

October 1, 1987 through November 30, 1987

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

Coagulation Studies Using Human Glomerular Endothelial Cells

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

PI:	M. Lange	Guest Researcher	MDB, NIDDK
Others:	L. Striker	Expert	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	T. Doi	Visiting Associate	MDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH
Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43214-04 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Cell and Molecular Biology of Glomerular Cells Derived From Transgenic Mice

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

PI:	K. MacKay	Medical Staff Fellow	MDB, NIDDK
Others:	L. Striker	Expert	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	S. Elliot	Biologist	MDB, NIDDK

COOPERATING UNITS (if any)

School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania (R. Brinster).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

3

PROFESSIONAL

3

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

Current models of glomerulosclerosis (GS) have yielded little information about the cellular and molecular abnormalities that are critical in the initiation and progression of this disease. The complexity of the kidney and glomerulus make isolation and examination of pure cultured populations of glomerular cells an attractive method for beginning to answer these questions. Unfortunately other models of GS involve extrarenal causes of glomerular injury. Because of this it is quite likely that glomerular cells isolated from these models would not maintain the abnormal behavior *in vitro* which led to the development of GS *in vivo*.

We have identified several lines of mice transgenic for the early region of simian virus 40 (SV40) that develop progressive glomerulosclerosis. As there are no evident extrarenal sources of injury, and since expression of the foreign DNA has been documented to occur in whole kidney we suspect that the glomerular disease may be secondary to expression of the foreign DNA by glomerular cells *in vivo*. We have isolated lines of glomerular endothelial, mesangial, and epithelial cells from transgenic mice and have isolated pure cultures of mesangial and epithelial cells from their normal litter mates. As preliminary data from the *in vivo* model indicates that proliferation of glomerular cells is an early event in the development of GS in transgenic mice we plan to begin the evaluation of several growth factors on the behavior of individual cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43217-04 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Renal Lesions in Leukemias, Lymphomas and Carcinomas

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

PI: L. Striker Expert MDB, NIDDK

Others: G. Striker Director DKUHD, NIDDK
L. Agodoa Medical Officer MDB, NIDDK

COOPERATING UNITS (if any)

National Cancer Institute, Bethesda, Maryland (M. Linehan and M. Merino).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

We have been examining the glomerular lesions in kidneys from patients who undergo a nephrectomy for renal cancer. In areas non-invaded by the tumor there is in half the cases examined a marked mesangial proliferation and occasional synechiae suggesting a glomerular disease which could be mediated by growth factors released by the tumor.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43218-03 MD

PERIOD COVERED

October 1, 1987 through November 30, 1987

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

Development of Human Glomerular Cell Lines

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

PI:	M. Lange	Guest Researcher	MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	L. Striker	Expert	MDB, NIDDK
	S. Elliot	Biologist	MDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL

OTHER:

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43219-03 MD

PERIOD COVERED

October 1, 1987 through November 30, 1987

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

Glomerular Endothelial Cells and Immune Complexes

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

PI:	M. Lange	Guest Researcher	MDB, NIDDK
Others:	L. Striker	Expert	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	L. Agodoa	Medical Officer	MDB, NIDDK
	S. Elliot	Biologist	MDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43220-03 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Regulation of Expression of Angiotensin Converting Enzyme in Renal Glomeruli

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

PI: L. Striker Expert MDB, NIDDK

COOPERATING UNITS (if any)

National Institute of Mental Health, Bethesda, Maryland (B. Martin);
Emory University, Atlanta, Georgia (K. Bernstein).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

.1

PROFESSIONAL:

.1

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

It has been suggested on an experimental basis that elevated intraglomerular pressure leads to glomerulosclerosis. The regulation of angiotensin converting enzyme (ACE) production plays a central role in maintaining normal intraglomerular pressure. This project is designed to isolate the gene encoding ACE from mouse kidney to further study the regulation and expression of the enzyme.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43221-03 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Biology of Insulin Receptors in Glomerular Cells

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

PI:	S. Elliot	Biologist	MDB, NIDDK
Others:	F. Conti	Visiting Fellow	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	L. Striker	Expert	MDB, NIDDK
	T. Doi	Visiting Associate	MDB, NIDDK

COOPERATING UNITS (if any)

Diabetes Branch, NIDDK (M. Lesniak)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

3

PROFESSIONAL:

3

OTHER:

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

We propose to study insulin specific binding on glomeruli from mice and humans. Binding of insulin to mesangial cells from normal mice is being investigated. The nature of the receptor will be studied and elucidated. Binding of insulin to an endothelial clone derived from normal mouse glomeruli has been shown. The eventual role of insulin as a progression factor is being investigated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43222-03 MD

PERIOD COVERED

October 1, 1987 through September 31, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)
Pathogenesis of Murine Lupus Nephritis

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

P. I.: H. A. Austin, Expert, MDB, NIDDK
 Others: J. E. Balow, Senior Investigator, MDN, NIDDK
 C. Cadena, Biologist, MDB, NIDDK

COOPERATING UNITS (if any)

Armed Forces Institute of Pathology, Washington, D. C. (Drs.
 Antonovych and Sabnis).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

Investigations of the pathogenesis and treatment of lupus nephritis are facilitated by the availability of inbred strains of mice that develop disease similar to human systemic lupus erythematosus. The natural evolution of the diverse histologic features of murine lupus nephritis is being studied to delineate the types of glomerular and tubulo-interstitial lesions as well as the characteristics of the immune deposits. Monoclonal antibodies and immunoperoxidase staining of frozen sections are employed to study the types and distribution of lymphoid cells in glomerular, vascular and tubulo-interstitial lesions. Changes in mesangial clearance of a macromolecular tracer, ferritin, will be related to these features of progressive glomerular disease. Ferritin is injected intravenously at various stages of the disease and the deposition of the exogenous protein is studied by light, immunoperoxidase and electron microscopic techniques. The impact of biologic response modifiers (eg. tumor necrosis factor and/or lipid A) on serologic and renal histologic features is being investigated. The goal is to develop a model of a flare of lupus nephritis which would facilitate further investigations of immunopathogenetic mechanisms. Innovative treatment strategies will be studied to refine our approach to this disease. Clinical, histologic and immunologic outcome parameters will be evaluated including detailed studies of renal morphology, and flow cytometry analysis of the characteristics of peripheral blood lymphocytes and splenocytes.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43223-03 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Crescentic glomerulonephritis

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

P. I.: J. E. Balow, Senior Investigator, MDB, NIDDK
 Others: H. A. Austin, Expert, MDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43224-02 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Membranous Lupus Nephropathy

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

P. I.: J. E. Balow, Senior Investigator, MDB, NIDDK
 Others: H. A. Austin, Expert, MDB, NIDDK

COOPERATING UNITS (if any)

Stanford University, Stanford, CA (Dr. B. Myers). Clinical Center
 (Dr. D. Webb; K. Joyce, E. Vaughan, Nursing). NIAMS (Dr. J. Klippel).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The efficacy and toxicity of three immunosuppressive drug regimens will be evaluated in patients with membranous lupus nephropathy over a 12 month study period. Detailed tests of renal function, glomerular permselectivity and kidney biopsy morphology will be conducted at the beginning and end of treatment. Patients with systemic lupus erythematosus, nephrotic range proteinuria and biopsy documented membranous nephropathy will be treated with alternate day prednisone and will be randomized to receive: a) no additional therapy (control group), b) intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) oral cyclosporin A up to 200 mg per square meter body surface area daily for a total of 11 months. Lupus disease activity, renal function tests and drug toxicities will be monitored closely. Analysis will include comparison of the number of favorable outcomes of glomerular function and pathology as well as drug-related toxicities appearing in each treatment group at the end of 12 months of study.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43225-01 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Glomerular Changes Due to GH and IGF-I

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

PI:	L. Striker	Expert	MDB, NIDDK
Others:	T. Doi	Visiting Associate	MDB, NIDDK
	L. Agodoa	Medical Officer	MDB, NIDDK
	F. Conti	Visiting Fellow	MDB, NIDDK

COOPERATING UNITS (if any)

University of Washington, Seattle, Washington (R. Palmiter); School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania (R. Brinster).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4

PROFESSIONAL:

4

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Increased glomerular size occurs in the presence of normal maturation following unilateral nephrectomy in humans and animals and in disease states such as diabetes mellitus. The glomeruli are morphologically and functionally normal following nephrectomy in rats unless the remaining renal mass is severely reduced, in which case progressive glomerulosclerosis ensues. The hormonal regulation of compensatory hypertrophy is not fully understood, however total kidney IGF-I mRNA levels are increased following unilateral nephrectomy. This suggests a role for this hormone in hypertrophy of the adult kidney as well as in normal development. There are abnormalities in the circulating levels of GH in some diseases associated with increases in glomerular extracellular matrix and cell number such as diabetes mellitus. The availability of transgenic mouse strains expressing elevated levels of IGF-I, GH, and GHRF provides an opportunity to study the renal effects of chronic hormone exposure. We have observed that mice containing an MT-I IGF-I fusion gene develop large glomeruli which are normal in appearance, whereas those transgenic for either growth hormone or growth hormone releasing factor have large glomeruli which are hypercellular, whereas progressive glomerulosclerosis develops later.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43226-01 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Effect of TGF-B on Glomerular Cells

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

PI: K. MacKay Medical Staff Fellow MDB, NIDDK

Others: L. Striker Expert MDB, NIDDK

G. Striker Director DKUHD, NIDDK

L. Agodoa Medical Officer MDB, NIDDK

T. Doi Visiting Associate MDB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS.

3

PROFESSIONAL:

3

OTHER:

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(a) Human subjects

(b) Human tissues

(c) Neither

(a1) Minors

(a2) Interviews

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

Proliferation of resident glomerular cells and the accumulation of mesangial matrix are histologic abnormalities which are observed in the course of many progressive glomerular diseases. We explored the potential regulatory effects of transforming growth factor-B (TGF-B) on these processes. We found that cultured mouse glomerular endothelial, mesangial, and epithelial cells as well as isolated rat glomeruli possess high affinity receptors for TGF-B. We also found that while TGF-B consistently inhibited the proliferation of glomerular endothelial and epithelial cells it acted as a bifunctional regulator of mesangial cell proliferation. The presence of TGF-B receptors on each glomerular cell type and on isolated glomeruli, the multiple potential sources of TGF-B within the glomerulus and the demonstrated responsiveness of cultured glomerular cells to TGF-B convine to suggest that potentially important interactions may occur between resident glomerular cells and TGF-B in vivo.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43227-01 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Role of IGF-I in the Biology of Mouse Glomerular Cells

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

PI:	F. Conti	Visiting Fellow	MDB, NIDDK
Others:	T. Doi	Visiting Associate	MDB, NIDDK
	S. Elliot	Biologist	MDB, NIDDK
	L. Striker	Expert	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK

COOPERATING UNITS (if any)

Diabetes Branch, NIDDK (M. Lesniak, J. Roth)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4

PROFESSIONAL:

4

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minor
- (a2) Interviews

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

The kidney has been shown to be a site of production of IGF-I in the rat, mouse, and human in many experimental conditions. IGF-I mRNA and peptide were found to increase in renal compensatory hypertrophy following partial nephrectomy suggesting a role of this peptide in renal growth. However the sites of action and the cell types responsible for IGF-I synthesis have not been identified.

To further investigate the role of IGF-I in the renal glomerulus we used mouse glomerular cells in culture. Separate cultures of glomerular endothelial, epithelial and mesangial cells were obtained from isolated glomeruli using patch cloning and dilute plating techniques. These cells were studied for the presence of IGF-I receptors, the mitogenic response to IGF-I and the synthesis of this growth factor. Glomerular mesangial cells were found to have IGF-I receptors and to be sensitive to the mitogenic action of IGF-I. These cells were also found to synthesize and release in the culture medium an IGF-I like molecule. Glomerular endothelial and epithelial cells were found to have IGF-I receptors, but no IGF-I like molecule was detected in their culture medium.

These data suggest a role for IGF-I in the maintenance and regulation of kidney structure and function. They demonstrate that glomerular mesangial cells may be a source of IGF-I in the kidney. IGF-I might be one of the growth factors locally released in the glomerulus acting in an autocrine and paracrine fashion.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43228-01 MD

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Biology of Human Glomerular Mesangial Cells

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

PI: L. Striker Expert MDB, NIDDK

Others: T. Doi Visiting Associate MDB, NIDDK
S. Elliot Biologist MDB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

4

PROFESSIONAL:

4

OTHER:

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

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

Mesangial cell hyperplasia is a feature common to several human glomerular diseases. The cause of this increased cell number is unknown. We assessed human mesangial cells in vitro and found that they possessed an insulin-like growth factor-I (IGF-I) receptor consisting of α and β units (Mr 130k and 90k respectively). Fifty percent inhibition of IGF-I specific binding to the receptor required $1 \times 10^{-9}M$ IGF-I, $\geq 1 \times 10^{-6}M$ insulin and $1 \times 10^{-7}M$ multiplication stimulating activity (MSA). Analysis of binding by the method of Scatchard revealed one type of IGF-I receptor with a $kd = 1.35 \times 10^{-9}M$, and a number per cell of 1.04×10^5 . Binding studies on whole glomeruli was of similar specificity and there were 7.17×10^7 receptors per glomerulus ($kd = 1.12 \times 10^{-9}M$). Examination of the effect of IGF-I on the cell cycle revealed that cells treated with platelet derived growth factor (PDGF) had a rapid 3H -thymidine response which was abolished by anti-PDGF antibody. Similarly, the labeling index of cells pretreated with PDGF, washed and then exposed to IGF-I was increased, whereas if the order of ligand exposure was reversed, there was no such additive effect. Finally, PDGF increased RNA and protein synthesis, which was not enhanced by IGF-I. In summary, human mesangial cells, and whole glomeruli, possess IGF-I specific receptors and IGF-I was found to act as a progression factor in the cell cycle.

The second Edelhoeh Memorial Lecture was presented by Dr. John Gwynne of the University of North Carolina School of Medicine. Dr. Gwynne, who had been a fellow with Dr. Edelhoeh from 1972 to 1974, spoke on cellular metabolism of HDL in the adrenals. The lecture was given in the Endocrine Grand Rounds of the Interinstitute Endocrinology Training Program.

Research fellows and scientists from abroad who took part in the Branch's research came from Greece, Brazil, India, Japan and Italy.

Dr. Robbins was co-organizer of a Fogarty Center Conference on Iodine and the Brain. The symposium was co-sponsored by the International Council for the Control of Iodine Deficiency Diseases and focussed on the neuropsychiatric aspects of iodine deficiency and the role of thyroid hormones in brain development and function.

The following scientists delivered invited lectures at several international meetings. Dr. Robbins spoke on protection of the thyroid against radioiodine exposure from nuclear accidents at the European Thyroid Association in Lausanne, Switzerland and on the effects of radioiodine fallout in the Marshall Islands at the Japan Society of Nuclear Medicine in Nagasaki. Dr. Wolff presented a lecture on iodine-lipid interactions in the thyroid, at the Henning Symposium in Holzhausen, Germany. He also attended the European Symposium on the Structure and Function of the Cytoskeleton and led a session entitled "Regulation of microtubule assembly by Ca^+ and calmodulin". Dr. Nikodem lectured on the mechanism of action of thyroid hormones at the International Congress of Endocrinology in Kyoto, Japan.

I. Thyroid Biochemistry and Pathophysiology

A. Thyroid hormone-Protein Interactions

In a study of the minor thyroid hormones-protein complexes in human plasma it was demonstrated that the circulating thyroxine (T_4) and triiodothyronine (T_3) are bound to all 3 classes of lipoproteins (VLDL, LDL, and HDL) with more than 90% bound to HDL. It was inferred that the binding was to the apolipoprotein moiety. This has now been confirmed by photoaffinity labeling of high density lipoprotein (HDL) isolated from fresh human plasma by ultracentrifugation ($d=1.063-1.210$). Polyacrylamide gel electrophoresis of HDL labeled with 0.5nM [$3,5\text{-}^{125}\text{I}$] T_4 demonstrated two radioactive bands. The larger had a molecular mass of 28 kDa and coincided with the major apolipoprotein of HDL, apoA-I. The smaller band (≈ 9 kDa) was either apoC-II or apoC-III. The second major apoprotein of HDL, apoA-II, did not bind T_4 . When photoaffinity labeling was done with excess T_4 ($1\mu\text{M}$), T_4 binding to apoA-I was reduced by 50-60%. Since labeling of isolated apoA-I was reduced even further (74%) by excess T_4 , the binding appeared to be inhibited by the lipids in HDL. This was confirmed by studying the non-covalent binding of [^{125}I] T_4 to apoA-I in the presence of various lipids; the strongest inhibition was exerted by unsaturated fatty acids, cholesterol, cholesterol esters and

phospholipids, in that order. These findings can explain the earlier observation that most of the lipoprotein-associated T_4 and T_3 in plasma is found with the smallest HDL molecules.

It was reported earlier (Grimaldi, Bartalena and Robbins) that thyroxine-binding globulin (TBG) isolated from human plasma is contaminated by a 66 kDa protein having two T_4 binding sites and two 27 kDa subunits, and that this "27 kDa protein" is synthesized in the liver. It has now been shown that the 66 kDa protein and the low molecular weight HDL are identical, and that the 27K protein is apoA-I. In addition to two moles of apoA-I, the 66 kDa protein contains cholesterol, lecithin and sphingomyelin in the molar ratio 5:8:2. This constitutes a previously unrecognized lipoprotein of human plasma.

The beta-lipoproteins (VLDL and LDL) were also shown to bind T_4 in the apolipoprotein moiety. Photoaffinity labeling showed that T_4 was associated with apoB-100 (550 kDa) and its proteolytic cleavage products, apoB-74 (410 kDa) and apoB-26 (140 kDa). When photoaffinity labeled with excess T_4 (1 μ M or 10 μ M), apoB-associated [125 I] T_4 was decreased by 40-52% or 53 to 87%, respectively. This is consistent with an affinity constant of LDL for T_4 of $2.5 \times 10^6 M^{-1}$ determined by equilibrium binding. Similar experiments with purified apoA-I gave an affinity constant of $7.5 \times 10^6 M^{-1}$ but purified apoB could not be studied because of its limited solubility. Lipoproteins enter certain cells by receptor-mediated endocytosis, thereby making cholesterol available for cell metabolism. This suggests that lipoproteins may also provide a special mechanism for cellular entry of thyroid hormones, despite the fact that lipoprotein-bound thyroid hormone represents only a minor fraction of the serum T_4 and T_3 . The validity of this hypothesis and its possible physiological importance is currently under study (Robbins, Benvenga, Cahnmann).

B. Thyroid Hormone Metabolism

In a continuing investigation of the mechanism of thyroid hormone transport into cells of the central nervous system, work has been extended on two cultured cell lines: human glioma Hs683 and mouse neuroblastoma NB41A3. Since glial cells (astrocytes) are a component of the blood-brain barrier, they could have a role in supplying T_4 to neurons, and it was shown previously that the glioma cells have a remarkably high T_4 uptake. Furthermore, T_4 transport into neurons is important since most of the T_3 bound to nuclear receptors is derived from intracellular monodeiodination of T_4 . The present experiments have shown that about 80% of the T_4 taken up by the glioma cells is saturable and dependent on metabolic energy. Kinetic analysis of the initial rate of uptake gave a K_m of 2.8 ± 0.6 (s.d.) nM which is similar to that of other CNS cells, but the maximum velocity (118 fmols/ 10^6 cells/min) was 10-fold higher. Most of this T_4 is accumulated in the cytoplasm and may be involved in transcellular movement of the hormone.

In the neuroblastoma cells it was found that T_4 uptake was much lower when the experiment was conducted in the culture medium (RPMI 1640) than in Hank's balanced salt solution. The components of the culture medium responsible for inhibiting T_4 uptake were found to be amino acids of the "L system", notably phenylalanine. Uptake of T_4 by the nuclear

receptors in the cells was also blocked, but not uptake into isolated nuclei. It was concluded that the transport of thyroid hormones, especially T_4 , across the plasma membrane may share the L system amino acid transport mechanism and that some of these amino acids are competitive with thyroid hormones at physiological concentrations.

An attempt to identify the T_4 transporter in the neuroblastoma plasma membrane was initiated. Preliminary results of affinity labeling with the N-bromoacetyl derivative of L- T_3 revealed three saturable radioactive bands, with apparent molecular masses of 24, 64 and 79 kDa. In contrast, a 29 kDa band showed labeling with BrAc-D- T_3 but not BrAc-L- T_3 . This stereospecific binding indicates that these proteins could be involved in hormone transport.

A novel class of thyromimetics (Underwood et al, 1986) have been shown to reduce cholesterol in rats with little effect on cardiac function. The effect of one of these compounds (SKFL-94901, 3,5-dibromo-3'-pyridazinone-L-thyronine) on T_3 transport was studied. Kinetic analysis of the initial rate of T_3 uptake into myoblasts (L6E9), hepatoma cells (HepG2) and neuroblasts (NB41A3) showed that L-94901 decreased the maximum velocity (V_{max}) in neuroblasts and hepatoma cells 2 to 2.5 more effectively than in myoblasts. This differential effect on plasma membrane transport of T_3 may explain the greater *in vivo* effect of this compound on cholesterol metabolism (Robbins, Lakshmanan, Goncalves, Cahnmann).

C. Thyroid Hormone Action

Regulated expression of thyroid hormone responsive genes is mediated by binding of the T_3 -receptor complex to specific DNA sequences. Recently, it was reported that c-erb-A proto-oncogenes encode thyroid hormone receptors. The T_3 receptor isolated from a rat brain cDNA library (rTR α) (Science 237:1610) showed a high degree of sequence similarity with previously characterized human and chicken steroid receptor cDNA (Nature 324:635 and 641). Additionally, Thompson et al. reported that this rTR α mRNA is abundant in brain and absent in liver, contradicting previously published findings that both tissue contain a low amount of T_3 receptor(s). In the present work, a rat brain cDNA library was screened with the ^{32}P -labeled 500 bp Pst I fragment of the viral gene, v-erb-A, and 12 positive clones were obtained. They were further characterized by restriction endonuclease mapping and sequence analyses as two sets of clones (vI, vII). The 1910 nucleotide sequence of the longer cDNA (vI) contains a long open reading frame encoding a protein of 454 amino acids with the ATG at nucleotide 78. The shorter cDNA (vII) starts at nucleotide 413 of vI and lacks 117 nucleotides from 1074 to 1190 corresponding to the amino acids 333 and 371, respectively, but is otherwise identical. Comparison of the predicted amino acid sequences of rTR α vI with the putative rat brain T_3 receptor revealed striking similarities in the presumed DNA-binding domain (100%), and in the first 180 amino acids of the T_3 -binding domain. The last 40 amino acids of rTR α are substituted by 122 and 83 amino acids in rTR α vI and rTR α vII, respectively. Thus, at least two variant forms, rTR α vI and rTR α vII of the T_3 receptor exist in the rat brain.

Nucleotide sequence analyses of the genomic clone obtained by screening a rat genomic library with the probe common to rTR α and its variants revealed that unique sequences of rTR α and vI and vII are present in the same clone. Pathways to generate rTR α vI and vII mRNAs by alternative splicing from the rTR α gene were proposed. The splice junction sequences conform to the GT/AG rule; i.e., spliced out sequences begin at the 5' end with the dinucleotide GT and terminate with the dinucleotide AG.

To determine whether the variant forms of rTR α encode a protein that specifically binds thyroid hormones, rTR α vI and vII were cloned into the pGEM 3 expression vector to allow in vitro transcription with the T7 RNA polymerase followed by translation in a reticulocyte lysate. Definite specific binding was detected for products of rTR α as previously reported by Thompson et al. However, neither T₃, thyroxine, nor reverse T₃ bound specifically to the translation products derived from rTR α vI or vII. This unexpected finding prompted an assessment of the levels of expression of rTR α and rTR α vI, vII mRNAs in rat brain. Various cDNA probes were used in Northern blot analyses of brain poly(A)⁺ RNAs. This indicated that in the brain, a 2.6 kb band corresponded to variant mRNAs, and a 5.4 kb band to rTR α mRNA. It was concluded that only the variant forms of c-erb-A mRNA are expressed at high levels (especially in brain) and therefore probes which do not discriminate between variant and receptor messages cannot be used to estimate their relative abundance. The physiological relevance of these variant mRNAs will be studied further with emphasis on their function during development and their possible involvement in the mechanism of T₃ action. The hypothesis that the variants are involved in regulation of the T₃-receptor gene will be tested (Nikodem, Mitsuhashi, Tennyson).

It was previously shown in cultured normal rat thyroid cells (FRTL-5) that TSH, acting through cAMP, increased the level of malic enzyme (ME) mRNA about 7-8 fold above the basal level. This increase was not due to transcriptional activation of the ME gene. It has now been demonstrated by measuring ³H-thymidine incorporation that the ME mRNA levels, reached a maximum at 16 h, corresponding to the G phase of the cell growth cycle. This declined to near the control level after 48 h, corresponding to the S phase. The rapid decline (within 24 h) indicated that the cells were well synchronized. Since TSH did not change the transcription rate of the gene, the increase in mRNA is likely to be related to mRNA stabilization. The responsible factor or factors appear to be proteins since cycloheximide prevented the TSH-induced rise (Nikodem, Grieco).

In a continuation of studies on the mechanism by which T₃ increases hepatic ME synthesis, experiments were performed to verify the earlier evidence that the hormone causes stabilization of nuclear ME mRNA. Two ME gene intronic probes were constructed which contained no repetitive sequences, did not cross hybridize with any ME exonic sequences, had no similarity to any cytoplasmic sequences, but did hybridize with ME nuclear RNA. These two probes, of about 1.5 kilobases, resided in introns 3/4 and 4/5.

Nuclear run off analyses using liver nuclei from control or T₃-treated rats showed that the rate of ME gene transcription was

stimulated by T₃ treatment. A maximal increase of ≈3-4 fold in ME nuclear mRNA synthesis was detected with any of the ME cDNA probes as well as with the two intronic probes, indicating that an additional mechanism(s) must be operating to account for the ≈ 12 fold increase in ME mRNA concentration after T₃ treatment.

The concentration of nuclear ME RNA hybridizing with the ME intronic probes as well as with ME cDNA of ≈ 3.0 kb was measured as a function of time after beginning T₃ treatment. All probes showed an elevation in nuclear ME mRNA which reached a maximal 12 fold-increase and was half-maximal at 60 hours. Since malic enzyme pre-mRNA rose at the same rate and by the same amount as mature mRNA, it was concluded that the information specifying a change in degradation caused by T₃ resides in the primary transcript. This assumes that the excised intronic sequences are rapidly degraded and thus do not contribute substantially to the hybridization signals. As a control, the effect of a high carbohydrate diet, which increases ME mRNA in cytoplasm without affecting either the transcriptional rate or nuclear RNA, was examined. As expected, the level of nuclear malic enzyme RNA after high carbohydrate feeding was the same as in control liver when determined with the two intronic probes. It was concluded that the T₃-induced cellular increase of malic enzyme mRNA in rat liver is due to stimulation of transcription of the gene augmented by stabilization of the primary transcript (Nikodem, Song).

Elucidation of the functional elements of the promoter region of the malic enzyme gene has continued with a series of experiments designed to determine the cis-acting regulatory elements necessary for basal transcription. Using a cell-free in vitro transcription system of HeLa cell extracts, it was found that maximal transcriptional activity was imparted by a 5' flanking region containing 881 base pairs proximal to the major transcription initiation site. Analysis of deletion mutants of the promoter revealed that there are at least 3 regions which contain positive cis-acting regulatory elements. These regions are: 1. a TATA box-containing the region from -881 to -355; 2. a consensus binding site for the AP-1/c-jun proto-oncogene family of transcription factors from -145 to -122, and 3. a unique 10 base pair direct repeat from -73 to -51. In addition, it appears that a negative cis-acting regulatory element lies within the region from -354 to -177. Furthermore, the promoter region of the ME gene differs from the sequences found in tissue specific promoters, but resembles promoters of housekeeping genes and thus could provide a different model in studying T₃ regulated gene expression than the often used growth hormone gene. Since the mechanism of expression for this class of promoters is poorly understood, the ME promoter region could be used in studying the basal level of expression of these genes as well.

It is believed that transcription of genes is mediated by the multiple control cis regions to which regulatory trans-acting factors bind and thus potentiate RNA synthesis in coordinate fashion. The first step toward identifying the mechanism(s) underlying transcriptional activity of a gene is the mapping of binding of the cellular trans-factors to the regulatory sequences. These specific interactions provide a basis for the purification of regulatory proteins and their biochemical characterization.

Gradual increases in the expression pattern of the ME gene suggest that the mechanism by which this gene is expressed might involve several regulatory elements. To identify these elements in the promoter region of the gene, gel shift mobility assays were used. The assay is based on the electrophoretic separation of protein-DNA complexes from free DNA in a low ionic strength nondenaturing polyacrylamide gel. The ^{32}P -labeled double stranded DNA fragment (.2ng) encompassing 142 bp of the ME promoter from -35 to -177 was incubated with $\approx 4 \mu\text{g}$ of nuclear proteins in the presence of 1.5 μg of poly dA.dT as a nonspecific inhibitor. Nuclear extracts were prepared from T_3 responsive (liver) and unresponsive (brain) tissue in order to investigate the effect of T_3 on nuclear binding proteins. The pattern of the binding complexes depended on the source of extracts. Furthermore, the intensity of some bands varied with the T_3 treatment. Treatment of hypothyroid rats with T_3 for 10 days (50 μg T_3 /100 g b.w. daily) increased nuclear protein binding in a fast migrating complex in liver but not in brain. A slower migrating complex seen as a broad band only in liver appeared to be oppositely affected by the hormone. This band was prominent in hypothyroid liver extract and almost absent after T_3 treatment; however, it was not always detected in different liver extracts, suggesting that its formation might involve a labile protein(s). Competition binding experiments with synthetic oligonucleotides revealed that the fast migrating complex mapped to the region of the 10 base pair direct repeat at position -73 to -51 of the promoter. Binding activity to this sequence was also present in other tissues and in HeLa cell extract, but only in liver was the binding enhanced by the hormone.

Since the ME gene is constitutively expressed and the direct repeat sequence is required for a basal level of expression of the gene, a factor interacting with this cis element would be expected to be present in all tissues. However, its elevated binding activity observed only in T_3 responsive tissue and modulated by thyroidal status might potentiate the tissue specific T_3 -stimulated expression of the ME gene in conjunction with other functional elements, especially the T_3 receptor. Current studies are addressing this possibility (Nikodem, Petty, Mitsuhashi, Morioka).

D. Studies in Thyroid Diseases

Serum thyroxine-binding globulin (TBG) concentrations measured by the Corning immunoradiometric assay in patients with nonthyroidal illness (NTI) had previously been found to be lower than those measured by radioimmunoassay. Because some of these patients were receiving newly recognized inhibitors of thyroid hormone binding - furosemide, diclofenac, fenclofenac, or mefenamic acid - it was decided to test whether the binding of labeled thyroxine to TBG (the principle of the kit) was inhibited by these drugs. Also tested were lipids, including free fatty acids (FFA), because most patients with NTI exhibit a thyroid-hormone-binding inhibiting activity in the circulation that apparently is related to unsaturated FFA. Among the lipids, unsaturated FFA were found to inhibit thyroid-hormone binding according to the degree of unsaturation. For nonlipid drugs the order of potency of inhibition was furosemide > diclofenac = mefenamic acid > phenytoin > heparin. It was concluded that the TBG Corning kit is a convenient and rapid method

for testing the inhibition of thyroid hormone binding. When measuring circulating TBG in NTI patients, however, a radioimmunoassay should be used, especially in those patients receiving furosemide.

A simplified low iodine diet was developed for outpatient use prior to I-131 scanning and therapy in thyroid cancer. Iodine intake of five subjects on the diet was measured by analysis of the urinary iodine/creatinine ratio before and after instituting the diet. The intake was decreased to approximately 50µg a day and this level was maintained for four weeks. The diet required only minimal instruction to be followed reliably. This level of iodine intake may increase radioiodine uptake in thyroid carcinomas.

Other studies that are in progress on patients with thyroid cancer include the following: 1) The effect of lithium carbonate on the secretion rate of radioiodine by thyroid cancer metastases. The purpose of this study is to improve the risk/benefit ratio of radioiodine therapy. 2) The effect of lithium carbonate on the secretion rate of radioiodine from thyroid remnants remaining after initial surgery for thyroid cancer. The purpose of this study is to improve the yield of complete ablation by low dose (30 mCi ¹³¹I) ablation therapy. 3) The management of patients on renal dialysis during radioiodine scanning and therapy. The purpose is to design safe and effective therapy in thyroid cancer patients who lack normal renal handling of radioiodine. 4) Effect of short term, profound hypothyroidism that occurs during radioiodine testing and/or therapy on a) postural hypotension and catecholamine responses and b) neuropsychiatric effects as determined by mood testing (Robbins, Lakshmanan, Benvenaga, Ain).

II. Mechanism of Cell Secretion

In a continuation of studies on the role of tubulin and microtubules in cell secretion, work has progressed on the physicochemical properties of the protein. The uncharged fluorescent dye, nile red, has been used to study hydrophobic subunit contacts in tubulin. The dye has a large separation of electron donor and acceptor functions, and therefore has a large dipole and is very sensitive to the polarity of the environment. It has a high quantum yield, is not sensitive to pH and has been shown to exhibit large intensity and wavelength shifts on partial denaturation of proteins, oligomerization of melittin, or Ca²⁺ binding to calmodulin. The interaction of nile red with pure rat brain tubulin shows a marked concentration-dependent blue shift (and intensity increase) which coincides with the known hydrodynamic equilibrium between the α and β subunits of the protein. Light scattering data are consistent with this interpretation. Equilibrium centrifugation data show some discrepancies, however. Part of this may be the result of limited viability of the protein in equilibrium runs and a computer hookup has been developed for short column runs on the centrifuge to facilitate analysis. This is now working satisfactorily and it should soon be possible to resolve the observed discrepancies.

In collaboration with Dr. Jay Knudsen (NHLBI), using time resolved laser fluorescence spectroscopy and phase-modulated fluorescence spectroscopy, two distinct life times of nile red fluorescence have been identified (with a possible third yet undecided). These appear to

correspond to the degree of exposure of the probe to the solvent. Stern-Vollmer quenching studies support such an interpretation.

Microtubule-stabilizing agents, especially 1M glutamate, tend to promote or stabilize the dimer and it is intended to explore the role of this equilibrium in the overall polymerization process.

The monomer-dimer equilibrium has also been approached through analysis of proteolytic accessibility. The α and β subunits of the tubulin dimer each possess a distal C-terminal subtilisin cleavage site which releases an acidic, small peptide. Cleavage rates, especially of β tubulin, are proportional to the subtilisin/tubulin ratio. However, if the rate constant increases due to decreasing tubulin, the extrapolated zero time intercept decreases. The decrease in zero time intercept is interpreted as being due to the appearance of a rapidly digested fraction upon dilution of tubulin, probably the monomer. The appearance of the fast fraction indicates a dissociation constant of about $1.5 \times 10^{-7} M$. This is lower than the value obtained from Nile red fluorescence measurements and the significance of this difference is now under study (Wolff, Sackett, Lippoldt).

III. Adenylate Cyclase of Bacterial Origin

One of the virulence factors of Bordetella pertussis is an extracellular, calmodulin-activated adenylate cyclase which can penetrate cells and there cause profound metabolism changes; e.g., paralysis of phagocytosis. There are several active forms of the enzyme which appear to be fragments of a larger precursor. The parent form has been cloned (by others) and has a molecular mass of 190 kDa. Not all of these forms can enter cells and it is not clear whether another protein may also be required; however, unpurified preparations can be used to study invasiveness. The problem of ATP leakage from cells as a source of cAMP was dealt with by use of a nonpenetrating form added to Y-1 mouse adrenal tumor, Chinese hamster ovary (CHO) and several other types of cells. This partially purified adenylate cyclase does not enter cells but, nevertheless, produces large amounts of cAMP in the medium as a result of the release of ATP by the cells. This could be directly measured, was reciprocally related to the cAMP produced, and was competed for by ATPases present in added serum, by hexokinase and, less effectively, by exoenzymes on the cell surface. The extent of ATP leakage varied widely between different cell lines, being marked in CHO and Y-1 adrenal cells but negligible in transformed lymphocyte lines. The uncertainty of the origin of cAMP found in media of cultured cells requires separate analysis of cell and medium cAMP and an assessment of ATP leakage.

Invasiveness was studied in several cell lines by exposure to adenylate cyclase-containing urea extracts of Bordetella pertussis (strain 114) organisms. This promotes the induction of high concentrations of intracellular cAMP. Accumulation is dose and temperature dependent, with significant accumulation occurring at $4^{\circ}C$, and is virtually instantaneous, with a doubling at 1 min. There is an absolute Ca^{2+} requirement. In Y-1 adrenal cells the urea extract adenylate cyclase stimulates steroidogenesis. Anti-B.pertussis antibodies inhibit cyclase activity, preventing further cAMP accumulation

after 10 min in cells previously exposed to urea extract, and the same effect is obtained by washing. This suggests that a portion of the cyclase is in a form not accessible to antibody or washing but accessible to substrate, and is probably internalized enzyme with a short life time. Continuing cAMP accumulation thus appears to require a continuing source of external cyclase. Since inhibitors of the effect of diphtheria toxin, such as NH_4Cl , methylamine, chloroquin or monensin, have no inhibitory effect on the urea extract induced accumulation of intracellular cAMP it is concluded that entry of the cyclase into cells is not by receptor-mediated endocytosis.

Charge may be involved in the penetration into CHO cells because it is maximally inhibited by polylysines with a minimum degree of polymerization (>6). Other polycations are also potent inhibitors. The adenylate cyclase itself shows a biphasic (stimulation - inhibition) response with a similar independence of polymer length above a certain minimum. Half-maximal inhibitory concentrations for cAMP accumulation correspond to half-maximal stimulatory concentrations of poly(L-lysine) for the cyclase. The inhibitory effect of polylysines on cAMP accumulation is not reversed by washing or enzymatic removal of neuraminic acid.

Limited proteolysis by trypsin, chymotrypsin or subtilisin show invasiveness to be far more sensitive than the catalytic activity of the cyclase. Whether this dissociation occurs on different domains of the same molecule or is due to differential hydrolysis of separate proteins is currently under investigation (Wolff, Raptis).

IV. Interaction of Proteins with Cell Membranes

It was shown earlier that the 110-100-50-47 kDa group of proteins associated with coated vesicles mediate the interaction of clathrin with the vesicle. Another associated protein (AP180) with an apparent molecular mass of 180 kDa was recently reported (Ahle & Ungewickell) and was shown to cause clathrin to polymerize into baskets under conditions where pure clathrin does not polymerize. This protein has now been purified by a simpler procedure and isolated coated vesicles have been shown to contain one copy of AP180 for each triskelion molecule. Sedimentation equilibrium analysis of this protein yielded a molecular weight of 115,000, indicating that its mobility on SDS gels is anomalous. To investigate whether it shares the same function as the other associated proteins in mediating the interaction of the vesicle with clathrin, vesicles stripped of most of the proteins and labeled with a fluorescent probe were studied. AP180, in the virtual absence of other APs, was capable of mediating the formation of the clathrin coat. This reaction of clathrin with AP180 was stoichiometric.

Previous work had shown that two types of baskets are formed from clathrin with average sedimentation coefficients of 150S and 300S. A third type having a sedimentation coefficient of 220S has now been demonstrated. The ratio of APs to clathrin determines the nature of the basket structure formed, with higher ratios giving smaller structures and lower ratios giving larger structures. By labeling APs and clathrin independently with fluorescent probes and polymerizing the baskets at pH

6.5 and 6.0, it was shown that the smaller structures are intermediates in the formation of the larger structures. It was also observed that APs aggregate under clathrin polymerization conditions in the absence of clathrin, implying that clathrin serves to keep these proteins soluble in the cell.

The combined group of proteins appear to determine the size of the structures formed from clathrin and may be responsible for the different sized baskets associated with various subcellular organelles such as the plasma membrane and the golgi apparatus.

Equilibrium centrifugation analysis and light scattering data on the newly discovered 27S intermediate polymer of clathrin (8S) in 3M glycol indicated that it is comprised of four triskelions. This was supported by deep etch electron microscopy of this polymer that showed the clathrin arms extending completely to form a closed tetrahedral structure (Prasad, Lippoldt).

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Thyroxine-Protein Interactions

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

PI: J. Robbins Chief CEB, NIDDK
 Others: S. Benvenga Guest Researcher CEB, NIDDK
 H.J. Cahnmann Scientist Emeritus CEB, NIDDK

COOPERATING UNITS (if any)

University of Messina, Italy (S. Benvenga).
 Dr. R.E. Gregg, Division of Intramural Research, NHLBI

LAB/BRANCH Clinical Endocrinology Branch

SECTION Hormone Metabolism and Action Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.8

PROFESSIONAL:

1.7

OTHER:

.1

CHECK APPROPRIATE BOXES)

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

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

We previously demonstrated that a portion of the circulating thyroid hormones in humans is associated with each of the plasma lipoproteins, VLDL, LDL and HDL. We now have shown that this interaction is the result of binding to apolipoproteins. In HDL, the major lipoprotein carrier, apoA-I and apoC-II and/or apoC-III account for this binding. In VLDL and LDL, binding is to apoB-100. The stereospecificity of the interactions are different from those of the other T₄-binding proteins in plasma, and the affinity is decreased by lipids. The latter accounts for the fact that the major lipoprotein carrier of thyroid hormone is a 68 kDa subfraction of HDL. This newly identified species was previously found as a contaminant in TBG preparations. The interactions of thyroid hormones with lipoproteins may provide a special mechanism for the entry of T₄ and T₃ into certain types of cells.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Structure of Polypeptide and Protein Hormones

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

PI: K. Prasad Visiting Associate, CEB, NIDDK

Others: R. E. Lippoldt Health Services Ofcr., CEB, NIDDK

COOPERATING UNITS (if any)

Washington University School of Medicine, St. Louis, MO (Dr. J.Heuser); Temple University School of Medicine, Philadelphia, PA (Dr. J.H.Keen)

LAB/BRANCH Clinical Endocrinology Branch

SECTION Protein Structure Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

.9

OTHER:

.1

CHECK APPROPRIATE BOX(ES)

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

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

The focus of this laboratory in the last few years has been the structural characterization of clathrin and clathrin coated vesicles that are involved in receptor-mediated endocytosis. Continuing our work on these lines, we have identified a new polymer of clathrin that is the smallest reported hitherto under specific buffer conditions namely 2mM MES, pH 5.9. This polymer has a sedimentation coefficient of 27S. Deep etch electron microscopy showed that this species is formed from clathrin triskelions (8S) with each triskelion extending its leg to join the globular end of the other triskelion. The extended nature of this triskelion leg is supported independently by Pearse's group at MRC, England, where they have shown that clathrin can form a cube like structure with a sedimentation coefficient of 42S. Equilibrium centrifugation of the 27S in 3M glycerol yielded a molecular weight of the polymer corresponding to four triskelions independently confirming the electron microscopic data.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies in Thyroid Diseases

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

PI: J. Robbins Chief, Clinical Endocrinology Branch CEB, NIDDK
 Others: M. Lakshmanan Medical Staff Fellow CEB, NIDDK
 M. Phyllaier Biologist CEB, NIDDK
 S. Benvenga Guest Researcher, CEB, NIDDK
 K. Ain Medical Staff Fellow, CEB, NIDDK

COOPERATING UNITS (if any)

University of Messina, Italy (Benvenga); Dr. J.Norton, Surgery Branch, NCI; Dr. J.Reynolds, Nuclear Medicine, CC

LAB/BRANCH Clinical Endocrinology Branch

SECTION Hormone Metabolism and Action Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.3

PROFESSIONAL:

1.1

OTHER:

.2

CHECK APPROPRIATE BOX(ES)

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

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

The reason for the low serum thyroxine-binding globulin (TBG) in nonthyroid illness (NTI) when measured by an immunoradiometric assay kit was investigated. We found that unsaturated free fatty acids, several non-steroidal anti-inflammatory drugs and the diuretic, furosemide, inhibit thyroid hormone binding. Although the kit can be used to test for binding inhibition, it should not be used to measure the circulating TBG concentration.

A simplified low iodine diet was developed for outpatient use prior to I-131 scanning and therapy in thyroid cancer. Iodine intake of five subjects on the diet was approximately 50 µg a day and this level was maintained for four weeks. The diet required only minimal instruction to be followed reliably. This level of iodine intake may increase radioiodine uptake in thyroid carcinomas.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 45014-17 CEB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Membranes and Secretion

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

PI: J. Wolff Associate Chief CEB, NIDDK

Others: D. L. Sackett Staff Fellow CEB, NIDDK
L. Knipling Technician CEB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been kept in abeyance pending the outcome of other studies on lipid-tubulin and lipid-binding protein-tubulin interactions. A new arrival is expected to start working on this project in Aug or Sept.

INACTIVE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Thyroid Hormone Secretion and the Function of Microtubules

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

PI: J. Wolff Associate Chief CEB, NIDDK

Others: D.L. Sackett Staff Fellow CEB, NIDDK

R.E. Lippoldt CEB, NIDDK

D. Zimmerman Guest Worker (2 mos) CEB, NIDDK

COOPERATING UNITS (if any)

Jay Knudsen, NHLBI

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.9

PROFESSIONAL:

1.3

OTHER:

0.6

CHECK APPROPRIATE BOX(ES)

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

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

Nile red, an uncharged, highly polarity-sensitive, fluorescent dye emitting in the red, has been used to study the interaction of the α and β subunits of pure tubulin. Time-resolved deconvolution of the fluorescence of liganded Nile red reveals two life times that correspond to two different emission maxima and quenching constants that suggest a solvent exposed and a solvent-shielded type of binding. Low tubulin concentrations cause a red shift and increased fraction of short life time fluorescence; high concentrations of microtubule stabilizing agent, especially glutamate reverse the process and cause a blue shift, increased life time and intensity. The equilibrium for this reaction corresponds to the hydrodynamic equilibrium constant for dimerization. Subtilisin susceptibility of the β subunit C terminus also reflects the monomer-dimer equilibrium in which this portion of tubulin is more exposed in the monomer. These are therefore relatively easy methods to assess the monomer/dimer equilibrium of tubulin and we expect to apply this analysis to overall microtubule assembly questions.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Adenylate Cyclase and Other Extracellular Products of B. Pertussis

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

PI: J. Wolff Associate Chief CEB, NIDDK
 Others: L. Knipling Technician CEB, NIDDK
 A. Raptis Visiting Fellow

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.7

PROFESSIONAL:

1.7

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

The penetration of the extracellular adenylate cyclase of Bordetella pertussis, a major virulence factor, into host cells has been shown not to occur by classical endocytotic mechanisms by which diphtheria toxin enters cells (e.g., inhibition by methylamine, monensin, chloroquin). There is, however, a charge dependent step in cyclase entry as shown by inhibition studies with polylysine. The minimum degree of polymerization of polylysine to attain inhibition is > 6 . The potency of longer polymers is a function of the number of lysyl residues yielding a constant I_{50} of $60\mu\text{M}$ lysine monomer concentration. This is not simply an inhibition of the added adenylate cyclase. Dissociation of invasiveness and adenylate cyclase activity has also been shown in studies with protease which suggest that invasiveness and catalytic activity either reside in different domains of the enzyme or on two separate proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
201 DK 45020-12 CEB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Synthesis of Thyroxine Transport Proteins

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

PI: J. Robbins Chief CEB, NIDDK

Others: M. Phyllaier Biologist CEB, NIDDK

COOPERATING UNITS (if any)

University of Pisa, Italy (L. Bartalena)

LAB/BRANCH Clinical Endocrinology Branch

SECTION Hormone Metabolism and Action Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

This project has been kept inactive this year pending the results of other studies being conducted in the laboratory.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Thyroid Hormone-Cell Interactions

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

PI: J. Robbins Chief CEB, NIDDK
 Others: M. C. Lakshmanan Medical Staff Fellow CEB, NIDDK
 A. Pontecorvi Visiting Fellow CEB, NIDDK
 M. Centanni Guest Researcher CEB, NIDDK
 M. Phyllaier Biologist CEB, NIDDK
 E. Goncalves Visiting Fellow CEB, NIDDK
 D. Foti Visiting Fellow CEB, NIDDK

COOPERATING UNITS (if any)

University of Rome, Rome, Italy (Pontecorvi, Centanni); University Rio Grande du Sul, Porto Alegre, Brazil (Goncalves); University of Catania (Foti)

LAB/BRANCH Clinical Endocrinology Branch

SECTION Hormone Metabolism and Action Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.0

PROFESSIONAL:

1.6

OTHER:

.4

CHECK APPROPRIATE BOX(ES)

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

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

A continuation of our work on the stereospecific, energy-dependent transport into cultured cells addressed several issues. Glioma cells were shown to have the most active plasma membrane transport of T_4 of any cells that we have examined. This was not accompanied by comparable uptake in the nuclei, suggesting a special role for thyroid hormone transport in these cells, possibly related to transcellular passage from capillary endothelium to neurons. In neuroblastoma cells, we demonstrated that plasma membrane transport of both T_4 and T_3 was inhibited by physiological concentrations of L-system amino acids, notably phenylalanine. A study of neuroblastoma membrane proteins by affinity labeling with bromoacetyl derivatives of thyroid hormones identified at least 2 proteins (24 kDa and 29 kDa) not previously found in other cell types. The 24 kDa protein reacted more strongly with L- T_3 and the 29 kDa protein with D- T_3 . A novel class of thyromimetics shown to have differential effects on liver and heart in vivo was found to exert a differential effect on T_3 transport into hepatoma cells and myoblasts. This may explain the effects observed in vivo.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Mapping of Triiodothyronine Responsive Genes

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

PI: V. M. Nikodem Visiting Scientist CEB, NIDDK

Others: H. Morioka Guest Researcher CEB, NIDDK

K. Petty Medical Staff Fellow CEB, NIDDK

T. Mitsuhashi Visiting Fellow CEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH Clinical Endocrinology Branch

SECTION Hormone Metabolism and Action Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.8

PROFESSIONAL:

1.7

OTHER:

.1

CHECK APPROPRIATE BOX(ES)

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

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

Elucidation of the functional elements of the malic enzyme promoter has continued with a series of experiments designed to determine the cis-acting regulatory elements necessary for basal transcription of this housekeeping gene. Using a cell-free in vitro transcription system of HeLa cell extracts, we found that maximal transcriptional activity from the malic enzyme promoter was imparted by a 5' flanking region containing 881 base pairs proximal to the major transcription initiation site.

Along with the aforementioned studies, we are also examining thyroid hormone regulation of transcription of the malic enzyme gene by studying the effect of thyroid hormone treatment on the binding of trans-acting factors to the malic enzyme promoter. Using gel mobility shift assays, we found that treatment of hypothyroid rats with high doses of triiodothyronine resulted in a greater than 2-fold increase in the binding of specific hepatic protein(s) to the unique 10 base pair repeat at -71. This binding activity was present in other rat tissues as well as HeLa and H35 cells but only in liver was the activity regulated by thyroid hormone indicating that the mechanism of thyroid hormone stimulation of malic enzyme gene expression in liver probably involves a tissue-specific up-regulation of specific transacting transcription factor(s). Current studies include identification, isolation, and cloning of the thyroid hormone-regulated transcription factors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 45034-05 CEB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Regulation of Specific Rat Liver mRNAs by Thyroid Hormone

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

PI: V. M. Nikodem, Ph.D. CEB, NIDDK
Others: D. Grieco, M.D. CEB, NIDDK
M. H. Song CEB, NIDDK
J.E. Rall CEB, NIDDK

COOPERATING UNITS (if any)

Dr. S.M. Aloj and Dr. L.Kohn, LBM, NIDDK

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.5

PROFESSIONAL:

2.4

OTHER:

.1

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

In FRTL-5 cells, thyroid stimulating hormone ($1 \times 10^{-11} M$) increases both malic enzyme mRNAs (21S and 27S) about 7-8 fold, 16 hours after the hormone addition. The cells are in the G phase. Since the rate of transcription was unchanged during the TSH incubation as determined by nuclear run on assay and since cycloheximide abolished the TSH effect, protein(s) are probably involved in the mechanism by which TSH increases the malic enzyme mRNA level via cAMP.

We have continued to study nuclear mechanisms by which thyroid hormone regulates malic enzyme (ME) mRNA in liver cytoplasm. The study included two ME intronic probes and nearly full size ME cDNA. These probes were used in nuclear run off and slot blot analyses of nuclear RNA preparations from euthyroid and T_3 treated rat liver. We conclude that T_3 activates transcription of the malic enzyme gene in rat liver and decreases the rate of degradation of pre-mRNA coding for malic enzyme. As a control, we examined the effect of a high carbohydrate diet which is known to increase malic enzyme mRNA without affecting either transcriptional rate or nuclear RNA (Proc Natl Acad Sci USA 83:4705). As expected, no change in the level of malic enzyme RNA in the nucleus was found with the intronic probes.

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Photoaffinity Labeling of Thyroid Hormone-Specific Binding Proteins

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

PI : V. M. Nikodem Visiting Scientist CEB, NIDDK

Others: H. J. Cahnmann Scientist Emeritus CEB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH Clinical Endocrinology Branch

SECTION Hormone Metabolism and Action Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 45037-03 CEB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Hormones and Cell Differentiation

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

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COOPERATING UNITS (if any)

None

LAB/BRANCH Clinical Endocrinology Branch

SECTION Hormone Metabolism and Action Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

This project has been kept inactive this year pending the results of other studies being conducted in this laboratory.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular biology of thyroid hormone receptors

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

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COOPERATING UNITS (if any)

None

LAB/BRANCH Clinical Endocrinology Branch

SECTION Hormone Metabolism and Action Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.2

PROFESSIONAL:

1.1

OTHER:

.1

CHECK APPROPRIATE BOXES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The nucleotide and predicted amino acid sequence of two novel variant cDNAs (rTR α , vI, vII), which we isolated from a rat brain cDNA library using the Pst I fragment of v-erb-A, showed virtual identity with the rat brain thyroid hormone receptor rTR α reported by Thompson, et al. (1987 Science 237:1610) in the putative DNA binding domain and in the first 180 amino acids of the hormone binding domain but no similarity except for 5 amino acids at the extreme 3' end.

Isolation and sequencing of the 3' end of the gene coding for rTR α , vI and vII mRNAs revealed that the 3' heterogeneity is due to alternative splicing of the primary transcripts of the same gene.

Northern blot analyses with probes unique to rTR α , rTR α vI, and rTR α vII showed that only the variant mRNAs are abundantly expressed in rat brain, contrary to the previously reported high-level expression of rTR α (ibid). Since in vitro translation products of rTR α vI, and rTR α vII did not bind thyroid hormones specifically, our findings explain the discrepancy between the reported abundance of the receptor mRNA and the low receptor levels determined by ligand binding studies in rat brain. These variant mRNAs are also expressed in kidney, heart, spleen, and liver, albeit at lower levels.

ANNUAL REPORT OF THE DIABETES BRANCH
NATIONAL INSTITUTES OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Introduction

The Diabetes Branch continues to pursue a broad based program which encompasses clinical research, studies on the mechanism of insulin action, with special emphasis on the nature and function of the insulin receptor, studies on the evolution of hormones and their function as messenger molecules, gene sequencing of insulin and insulin-like growth factors I and II, studies on morphological interaction of hormones with cells, and detailed studies of the biosynthesis of the insulin receptor.

A major feature of the preceding 12 months has been the resurgence of new studies of type II diabetes, especially involving new tools to investigate the disease in Pima Indians and other populations heavily affected by type II diabetes.

Recognition of Previous Achievements

Several grants were awarded to members of the Diabetes Branch: Juvenile Diabetes Foundation; American Diabetes Foundation, both the national organization and local affiliate; Diabetes Foundation; the Upjohn Company; a National Research Service Award; a Fellow of the Pharmacology Research Associates Program of the National Institute of General Medical Sciences; and an International Research Fellowship Award of the Fogarty International Center. In addition, one of the incoming Fogarty Scholars in Residence, (Professor Lars Terenius) will be spending his term in the Diabetes Branch. In addition, the alumni of the Branch have continued to distinguish themselves including two who began their research careers here: Robert J. Lefkowitz, Duke Professor of Medicine (at Duke University), was elected to the National Academy of Sciences and is outgoing president of the American Society for Clinical Investigation; C. Ronald Kahn, Iacocca Professor of Medicine at Harvard and Research Director of the Joslin Clinic, who is the incoming president of the American Society for Clinical Investigation, was a plenary lecturer at the quadrennial meeting of the International Endocrine Society and will receive the Diaz-Cristobal Prize for his work (started at NIH) at the triennial meeting of the International Diabetes Federation. Dr. Masato Kasuga was awarded the first young investigator prize of The Japan Diabetes Society for his work (started at NIH). Another one of the Diabetes Branch alumni, Lluís Bassas, received a Spanish award for outstanding basic science publication on a chick embryo project which was supported by a U.S.-Spain Cooperative Project grant (Diabetes Branch is the U.S. party). Another Spanish Fellow is being supported in part by the same U.S. Spain Cooperative Project. Jesse Roth is continuing service as a board member of the Weizmann Institute in Israel and completed a one year term as a consultant for MacArthur Foundation. He was also distinguished by being a Section Chairman for the Juvenile Diabetes Foundation World

Assembly on Diabetes in Monaco; AOA lecturer for the University of Puerto Rico; Plenary Speaker for Workshop for Monokines and Crytokines for the Gordon Conference on Immunology, Kroc Lecturer at MIT; Lazarow Lecturer at Minnesota Medical Foundation; and Keynote Speaker for the International Symposium on Obesity in experimental animals in Buckingham, U.K. The Diabetes Branch joined the Institute as host to US-Italian meeting on Diabetes in Bethesda.

RECEPTORS FOR INSULIN AND RELATED HORMONES

Tissue-Specific Differences. The alpha-subunit of the insulin receptor is the hormone binding subunit of the receptor and the beta-subunit is a protein kinase, capable of phosphorylating itself and a number of exogenous substrates. Many details of this phosphorylation reaction continue to be studied in fresh and cultured cells of human and murine origin derived from many tissue types including blood, nervous system, and liver. The insulin receptors of every tissue appear to have both a binding (α) and protein kinase (β) subunit and in that sense, all insulin receptors are similar; they are somewhat different, however, in their molecular weight based on migration on polyacrylamide gel electrophoresis. In studies carried out on receptors from rats, guinea pigs, chickens, lizards, alligators, and frogs, it is clear that these differences in structure are maintained both ontogenetically and phylogenetically. Further, similar studies have been carried out in neuroblastoma cell lines from adult rat tissue as well as primary cultures of rat neuronal and glial cells. The IGF-I receptor, like the insulin receptor, has both a hormone binding (α) and phosphorylating (β) subunit and differences among tissues that are quite analogous to those differences found among insulin receptors.

Possible Target for Insulin Action in Liver. We previously identified a Mr-120 kDa glycoprotein (pp120) in rat liver membranes which can be phosphorylated on tyrosine-residues by solubilized insulin receptors in vitro. This protein, designated pp120, fulfills the necessary criteria to be considered as a physiologically relevant substrate for the insulin receptor-associated tyrosine kinase. First, it can be phosphorylated directly by the insulin receptor in a cell-free system; and second, insulin stimulates tyrosine-specific phosphorylation of pp120 in intact target cells for insulin action.

Recent studies, in collaboration with investigators from Howard University, have identified pp120 as HA4, a 110kDa membrane glycoprotein of the bile canalicular domain of the hepatocyte. Monoclonal antibodies to HA4 were used to identify it as a substrate of the insulin receptor kinase. Anti-pp120 and anti-HA4 were found to cross-react, and phosphopeptide maps for each of the corresponding antigens were identical. The identification of pp120 as HA4 serves to link insulin action through the receptor tyrosine kinase activity to bile metabolism, and raises questions pertaining to the intracellular site(s) of action of the insulin receptor.

Insulin receptors are present in liver of rat fetuses in mid-gestation as early as day 14 of fetal development (full term is

22 days). Insulin stimulates autophosphorylation of the beta-subunit of insulin receptors prepared from rat livers of fetal (> 14 days of gestation) and neonatal rats. In contrast, insulin-stimulated phosphorylation of a Mr 120 kDA glycoprotein derived from rat liver membranes (pp120) is not observed in fetal liver until day 17 of gestation. Thereafter phosphorylation of pp120 (day 17) corresponds to the onset of several important differentiated metabolic functions in liver including glycogen metabolism and bile synthesis and secretion. The data suggest that maturation of the insulin receptor kinase occurs soon after initial appearance of the receptor in mid-gestation, but insulin-stimulated phosphorylation of endogenous substrate(s) is dependent on the appearance of substrates, such as pp120.

Possible Defect, Type II Diabetes. Our earlier investigations of Type II diabetes led to the discovery of a generalized defect in the relationship of the alpha and beta subunit functions in untreated patients. Specifically, the amount of kinase activity stimulated by insulin per unit of insulin binding is decreased in these patients compared to normals or obese non-diabetics. These data may indicate a defect in tyrosine kinase or in the interaction of the two subunits of the insulin receptors of these patients. We are pursuing these observations through studies of the subunit interactions, i.e., effects of reductants which break the covalent attachments between the subunits.

Other Studies of Patients. We have recently extended our studies to another disease with extreme insulin resistance, total lipodystrophy. In our preliminary studies from one patient, the dose response to insulin of the tyrosine kinase activity of the insulin receptors is diminished. This abnormality is not altered by fasting. In contrast, regulation of receptor number, physical size of the subunits and recognition by anti-human insulin receptor antibodies appear to be normal. We plan to study several of these rare patients to extend these observations.

Biosynthesis of Receptors for Insulin and IGF-I. The insulin receptor (like the receptor for insulin-like growth factor I) is an integral membrane protein composed of two major subunits, α and β of apparent molecular weights of 135,000 and 95,000, respectively. The α and β subunits of the receptor contain oligosaccharide side chains of the complex and high mannose types. With cultured cells pulse-chase labeling studies with radioactive sugars and amino acids followed by immunoprecipitation with anti-receptor antibodies and analysis on SDS/polyacrylamide gel electrophoresis have demonstrated the existence of a single polypeptide chain precursor of the insulin receptor, i.e., a proreceptor with an apparent molecular weight of 190,000. Our model for the biogenesis of the insulin receptor proposes that the single chain polypeptide precursor is translated and the high mannose core is added co-translationally to the nascent polypeptide in the endoplasmic reticulum of the cell.

Experimental manipulations that prevent removal of glucose from core oligosaccharides retard processing of the insulin receptor

and produce a marked decrease in cell surface receptors. However, proteolytic cleavage of the proreceptor is not blocked, and further processing of some of the carbohydrate chains is not completely inhibited. Furthermore, the processed receptors are inserted into the plasma membrane and their binding affinity is normal despite the presence of an undetermined number of glucosylated chains. In addition to carbohydrates, several membrane receptors contain covalently linked fatty acids. Both [³H]myristic acid and [³]palmitic acid are found attached to the insulin receptor subunits. The incorporation of fatty acids into the insulin receptor is dependent on protein synthesis and is also detected in the M_r=190,000 proreceptor form. Fatty acylation is thus a newly identified post-translational modification of the insulin receptor.

Effects of Glucocorticoids. Previously we have shown that inclusion of glucocorticoids in the culture medium increase the insulin receptor number in IM-9 lymphocytes. This increase was due to increased proreceptor biosynthesis. More recent studies have shown that this increase in receptor number is also associated with an increase in mRNA levels and the increased mRNA is secondary to increase transcription of the insulin receptor gene.

Defects in Syndromes of Extreme Insulin Resistance

The Diabetes Branch since the early 1970s has been the leader in defining disorders characterized by extreme insulin resistance, especially those centered on the insulin receptor. The two broad categories are those patients with congenital defects involving the insulin receptor (and early steps beyond the receptor) and those with autoantibodies directed against the receptor. While these syndromes have an intrinsic interest of their own, they also provide major new insights which should prove profitable in our attempts to understand the more common varieties of diabetes, especially Type II diabetes in adults, both thin and obese.

We have identified several classes of patients, each of which appears to have a different mechanism of insulin resistance. Some patients with genetic forms of extreme insulin resistance have quantitative receptor defects; the cause of insulin resistance is a marked (> 90%) reduction in the number of cell surface insulin receptors.

As described above, insulin receptors are composed of two major glycoprotein subunits [apparent molecular weight (M_r) of 135 kDa and 95 kDa], which are both derived from a common precursor molecule with M_r of 190 kDa. In one patient there was a marked reduction in the biosynthesis of both the 190-kDa precursor and the mature receptor, i.e., the defect appears to occur early in the biosynthetic pathway. In contrast, in two sisters with type A extreme insulin resistance, biosynthesis of the 190-kDa precursor proceeds at a normal rate. However, there appears to be a defect subsequent to the biosynthesis of the 190-kDa precursor, but before the insertion of the mature receptor in the plasma membrane. These data suggest the existence of at least two distinct types of biosynthetic defects which may give rise to a marked reduction in the number of insulin receptors on the cell surface.

Syndromes of Extreme Insulin Resistance Molecular Biological Studies. We have identified several patients with genetic forms of insulin resistant diabetes mellitus, in whom the disease is caused by mutations in their insulin receptor genes. In the patients who have been investigated thus far, we have identified different mutations in each kindred:

1. Decreased levels of insulin receptor mRNA. One patient with leprechaunism (leprechaun/Minn.1) had a 90% reduction in the level of insulin receptor mRNA in a cell line established by transformation of her lymphocytes with Epstein-Barr virus. This phenotype probably results either from impaired transcription, defective splicing, or decreased mRNA stability. Genomic cloning is being carried out to identify the mutation(s) in this patient.

2. Impaired insertion of receptors in plasma membrane. Two sisters with type A extreme insulin resistance had normal levels of insulin receptor mRNA despite markedly decreased numbers of insulin receptors on the surface of cultured lymphoblastoid cells. Analysis of restriction fragment length polymorphisms were consistent with the hypothesis that the mutation causing diabetes was genetically linked to the insulin receptor gene. cDNA cloning demonstrated a point mutation in the sequence encoding the receptor's α subunit. This missense mutation seems to impair the transport of the receptor for insertion into the plasma membrane.

3. Abnormal receptor molecules. Another patient with leprechaunism (leprechaun/Ark-1) is a genetic compound who is heterozygous for two distinct point mutations. One allele has a nonsense mutation at codon 672 which truncates the receptor near the C-terminus of the α subunit. The truncated receptor lacks a transmembrane domain and is not expressed at the cell surface. The other allele has a missense mutation leading to the substitution of glutamic acid for lysine at position 460 in the receptor's α subunit. This mutation impairs the ability of acid pH to dissociate insulin from its receptor. Associated with this defect, the receptor appears to be impaired in its ability to recycle to the plasma membrane after it is internalized by the cell.

OTHER STUDIES OF HUMAN DISEASE

New studies of Type II Diabetes in Pima Indians. The Branch has had a long standing commitment to the study of type II diabetes, its pathogenesis and its etiology. Along these lines several new projects have been initiated as well as previous ones extended. Elsewhere we have discussed studies of insulin-induced tyrosine phosphorylation defects in patients with diabetes as well as the molecular characterization of specific genetic defects associated with the insulin receptor. We have started new initiatives directed at understanding glucose utilization in brain. Studies of a group of patients from the Pima Indian tribe have been completed using the latest techniques including PET scanning with fluorodeoxyglucose and insulin clamp. In addition, the insulin receptor, insulin, and other components of the glucose regulatory system from the Pima Indians and from other groups with high prevalence of diabetes are under study. Further, possible auto-antibodies directed against these components are being searched for as well.

Animal model of Type II Diabetes. To complement studies in patients are studies on transgenic mouse that have multiple copies of the human insulin gene intergrated into their genome which they overexpress. This model in which hyperinsulinemia is a fundamental feature provides new insights for the role of insulin in early development as well as the role of hyperinsulinemia in the emergence of obesity or diabetes.

Hypoglycemia associated with non-islet cell tumors. We previously reported elevated levels of IGF-II-like material in plasma in about one third of patients with hypoglycemia associated with non-islet cell tumors. Recent studies of several tumors revealed high levels of IGF-II (measured by radioreceptor assay) and IGF-II mRNA in extracts of these tumors, further strengthening the association between IGF-II and tumor hypoglycemia.

Acromegaly. Acromegalic patients have continued to be followed following pituitary irradiation. Further, we are evaluating the effects of transsphenoidal hypophysectomy followed by irradiation and comparing them to either treatment modality alone. A group of patients have been followed to determine the effect of joint disease as a function of time following pituitary irradiation. It appears that the joint disease is a function of the age of the patient and to the degree of joint involvement at the time of initial therapy. Thus in patients with relatively severe disease, the joint disease progressed in spite of very significant reductions in growth hormone that occurred following radiation therapy. Other studies underway are attempting to determine the effect of pituitary irradiation on possible brain function and possible other complications.

We have studied the use of the long-acting somatostatin analogue, SMS 201-995 to inhibit abnormal hormone production, in patients with acromegaly, patients with TSH secreting pituitary tumors and those with glucagonomas. These studies have defined 1) an appropriate dosage schedule that controls TSH secretion by TSH secreting pituitary adenomas and the resultant hyperthyroidism, 2) an appropriate subgroup of acromegaly patients in whom this analogue, given thrice daily, controls GH hypersecretion, 3) the effects of the drug in glucagonoma syndrome in terms of control of glucagon hypersecretion and correction of hypoaminoacidemia. The results of our studies thus far show that the SMS 201-995 is especially effective in treatment of TSH secreting pituitary adenomas.

Morphologic Studies of Ligand Binding to Human Cells

This work represents over 11 years of collaboration between the Diabetes Branch and the Institute of Histology and Embryology at the University of Geneva. The initial observations demonstrated that polypeptide hormones are taken up by the cell through a process of receptor-mediated endocytosis similar to other biologically important ligands that bind to the cells. In the present study we find that when cells are incubated at 15°C, labeled insulin becomes less dissociable as a function of duration of incubation. This lower degree of dissociability correlates with the anatomical finding of an increasing localization of the ligand in coated pits.

Thus, it seems possible that this anatomical redistribution may account for two different dissociation rates and could explain the non-linear or non-exponential dissociation of insulin from its receptor.

Using an E-B virus transform lymphocyte cell line which we had shown previously to have insulin receptors with altered or slowed dissociation characteristics, we found by electron microscopy that a higher portion of ^{125}I -insulin bound to the non-villous surface of the cell. This result suggested that there are fewer villous projections rather than preferential binding to the non-villous surface. This represents the first anatomical defect in a cell line from a patient with insulin resistance.

In further studies we have investigated the role of receptor mediated endocytosis in insulin resistant states. Several groups of patients have been studied including non-obese type II diabetics, obese type II diabetics, lipotrophic diabetics and non-diabetic obese individuals. An interesting finding is that internalization or endocytosis is markedly impaired in the insulin resistance patients at 30 minutes of incubation but returns to normal by 60 minutes of incubation. This is in contrast to the type I or insulin dependent diabetic where internalization is impaired throughout the course of binding study, i.e., at 30 and 60 minutes. In the obese group the results are ambiguous.

Coated pits, indentations in the cell surface decorated by a bristle coat covered by the protein clathrin, has been the major path through which most ligands are taken up by cells, i.e. receptor-mediated endocytosis. A much larger number of smaller non-coated invaginations exist on the cell surface and these invaginations are preferred sites for binding of a ligand such as cholera toxin. We have now shown that the non-coated invagination is the primary mode by which cholera toxin is endocytosed by 3T3L1 cells that that immediately following internalization the two ligands enter the same vesicular compartment and are then passaged through the same endosomes and lysosomes. Thus two ligands which are dissimilar are internalized through two different types of structures and are endocytosed into common internal compartments once inside the cell.

RECEPTORS FOR INSULIN AND INSULIN-LIKE GROWTH FACTORS (I and II) IN BRAIN/CNS AND EMBRYOS

Rat CNS Receptors. Specific insulin receptors are widely distributed throughout the rat brain. The brain receptors are very similar to insulin receptors previously characterized in other tissues.

Applying autoradiographic techniques, we have studied the binding of ^{125}I -IGF-I and ^{125}I -IGF-II to brain receptors, demonstrated the specificity of binding, and compared the IGF binding patterns to those of insulin throughout the brain. Especially dense were regions similar to those of insulin i.e. choroid plexus, olfactory bulb, limbic regions, and cerebellum. In each area, the binding of each of the three peptides conforms to well defined cytoarchitectonic boundaries. However, each of the three peptides binds to a distinctive region within each area. Thus, except for the choroid plexus, it appears that the receptors

for the three peptides are binding to nearby but distinctly different groups of cells.

To determine whether the brain insulin receptor is unique to central nervous system tissues, or whether all neural tissues express this type of receptor, we examined retinal tissues and peripheral nerves. In peripheral nervous tissues the insulin receptor is similar to insulin receptors on liver, adipocytes, and placenta namely, its apparent Mr on SDS-PAGE is larger than that of the brain insulin receptor. Retinal insulin receptors had both "brain-type" and non-brain type insulin receptors.

Since insulin and IGF-I receptors are similar in structure, it was important to distinguish these receptors on the nervous tissues being studied in order to dissect out the function of each receptor. Having previously characterized the insulin receptor on neuronal and glial cells, we investigated the IGF-I receptors on these primary cultured cells from 1 day old neonatal rats. The IGF-I receptors on neuronal cells demonstrated an apparent Mr on SDS-PAGE similar to brain IGF-I receptors namely 10 kDa less than that of IGF-I receptors from placenta. Glial cells, on the other hand, express IGF-I receptors with apparent Mr similar to that of peripheral, non-neural tissues. These differences among IGF-I receptors are reminiscent of the differences in Mr of the insulin receptor from the same cell types i.e. neuronal versus glial cells. In these cultured cells IGF-I stimulated thymidine incorporation in a dose dependent manner, suggesting that IGF-I may play a role in growth of both neuronal and glial cells. Insulin on the other hand stimulated glucose uptake in glial cells and inhibited catecholamine uptake in neuronal cells suggesting that it had different functions from that for IGF-I.

Developmental Biology.

Offspring of diabetic mothers suffer a higher incidence of congenital malformations. Early studies by us and others in several types of early embryos in several model systems of growth and differentiation have suggested an important role for both insulin and insulin-like growth factors.

Chick Embryos. In studies with chick embryos, insulin appears to be required for normal development even before emergence of pancreatic endocrine function. Further, most if not all insulin-related receptors are present in tissues analyzed during organogenesis. We recently showed that anti-insulin receptor antibodies severely impaired embryonic growth. The effects are similar but not identical to the effects we observed after application of anti-insulin antibodies to the same early stage embryos. In recent studies, autoradiographic techniques have provided enough sensitivity to localize insulin receptors, as well as IGF I receptors, in embryos throughout gastrulation and neurulation.

The developmental expression of the insulin gene has been studied. Insulin mRNA is detected prior to pancreatic maturation in whole embryos and in at least one nonpancreatic tissue, the liver. In addition, we are using the eye lens to study insulin effects on

differentiation in an avascular tissue. The study of insulin action on δ -crystallin gene expression regulation may prove a useful model to understand further the actions of insulin in development.

Xenopus Laevis Embryos. In order to understand the role of insulin (and of insulin-like growth factors) and their receptors in early development, we have turned to the amphibian, *Xenopus laevis*, a major model of vertebrate development for further study. As part of the ground work, we have isolated the insulin from pancreas and have found two distinct insulins which to our surprise more closely resemble avian and mammalian insulins than they do fish insulins (or reptile insulins) in their amino acid sequence, and immunological and biological properties. The sequences have been confirmed by molecular cloning of both insulins which have been shown to be non-allelic, i.e. individual frogs contain both insulins. This work represents the early foundation for further studies in very early *Xenopus* embryos.

Rat Embryos. In view of the possibility that the insulin-like molecules may be more similar to molecules such as insulin-like growth factor-I (IGF-I), than insulin itself, we have cloned the rat IGF-I cDNAs for use as a hybridization probe to screen the various banks described above. These data revealed the high degree of conservation of IGF-I nucleic acid and amino acid sequences among mammalian species, and the characterized clones provide an array of probes with which to screen banks from unrelated organisms for the presence of insulin-like sequences, which may not be detectable with probes for insulin itself. As part of the characterization of these rat IGF-I cDNAs we have obtained preliminary evidence that one form of IGF-I mRNA may be subject to a novel form of translational control due to the presence of an extended inverted repeat sequence involving an alternate 5'-untranslated region sequence and the common 3'-untranslated region sequence. The appearance of the messenger molecules for these peptides in developing rat embryo are under investigation.

UNIFICATION HYPOTHESIS

The existence in invertebrates, unicellular eukaryotes, and prokaryotes of materials that resemble several vertebrate peptide hormones led to the suggestion that these peptide messengers may have arisen earlier in evolution than had previously been thought and may provide a greater role for cellular communication systems than had been suspected before. Consistent with this hypothesis, we reported material in plants (spinach and *Lemna*) that is very similar to mammalian insulin, yet distinctive. The role of this insulin-like material in plants is unknown but its existence is consistent with an early evolutionary origin of the insulin messenger peptide family. Alternatively, we cannot exclude a later convergent development of this family or incorporation of vertebrate DNA into plants.

We also reported previously (and others have confirmed), that plants contain material that closely resembles somatostatin, a

neuropeptide and hormone-like messenger molecule of vertebrates. As an extension of this work we undertook a large scale purification of the somatostatin-like materials from spinach. The first stage included a mass large scale purification from 10,000 pounds of spinach of the somatostatin related material. Both immunoreactivity of the N-terminal of the molecule and of the C-terminal of the molecule were detected. In more recent studies using HPLC and immunoaffinity chromatography, we have purified the N-terminal activity another 1000-fold and have freed it of the C-terminal activity. The N-terminal activity is being purified further in collaboration with Dr. Wyle Vale at the Salk Institute and his colleagues. The C-terminal activity is being purified further by former NIH colleagues who are now in Israel.

In further studies in this vein, we have been extracting microbes, specifically *Sacchomyces cerevisiae* and *E. coli* where insulin-related molecules and ACTH molecules respectively have been characterized, using a wide range of current techniques.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47001-07 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Phosphorylation of the Insulin Receptor

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

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	A. Ota	Visiting Fellow	DB/NIDDK
	G.L. Wilson	Biologist	DB/NIDDK
	Z. Shen-Orr	Guest Researcher	DB/NIDDK
	H. Werner	Guest Researcher	DB/NIDDK

COOPERATING UNITS (if any)

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LAB/BRANCH

Diabetes Branch

SECTION

Section of Molecular and Cellular Physiology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5.5

PROFESSIONAL:

4.5

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Brain insulin receptor and insulin-like growth factor I (IGF-I) receptors are similar to their peripheral, non-neural counterparts, being comprised of two alpha subunits and two beta subunits in a heterotetrameric formation. However, they have smaller apparent Mr compared to the peripheral receptors on sodium dodecylsulfate-polyacrylamide gel electrophoresis.

These unique insulin and IGF-I receptors have been studied in membranes prepared from whole retina, brain, peripheral nerves, as well as from neural-derived cultured cells. Primary cultures of neuronal cells contain unique insulin and IGF-I receptors resembling those of whole brain. Peripheral nerves and glial cells on the other hand, contain insulin and IGF-I receptors similar to those found in non-neural tissues.

In intact cells both insulin and IGF-I receptors undergo ligand-induced auto-phosphorylation as well as phosphorylation of endogenous substrates. Neuronal and glial cells in primary culture demonstrate rapid receptor autophosphorylation. NG 108, a neuroblastoma cell line, in addition to receptor phosphorylation demonstrates phosphorylation of an endogenous substrate phosphoprotein (pp 185) of Mr 185kDa. Both insulin and IGF-I induce phosphorylation of their receptors and pp 185 on tyrosine residues. Thus neural-derived cells express functional insulin and IGF-I receptors, and are model systems for studying insulin and IGF-I action on the nervous system.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47002-01 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Insulin Gene Expression and Insulin Action

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

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COOPERATING UNITS (if any)

LAB/BRANCH

Diabetes Branch

SECTION

Receptor and Hormone Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

8.7

PROFESSIONAL:

5.7

OTHER:

3

CHECK APPROPRIATE BOX(ES)

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

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

Molecular biological techniques are being used to discern gene expression of insulin, insulin-like growth factors and their receptors in several vertebrate model systems. Insulin is apparently a requirement for normal development, and the chicken embryo is one of the suitable models for studying the role of insulin in development. The pattern of expression of insulin gene in developing chicken pancreas and in whole embryo at prepancreatic stages is being undertaken. The role of insulin in cell differentiation and gene expression in the eye lens of chick embryo is also being used as a cell model to understand the action of insulin in development.

The amphibian, Xenopus laevis, is also a model system used to study development. Previously the amphibian insulin sequenced was unknown. In this laboratory insulin from pancreas of Xenopus has been isolated and its sequence characterized and confirmed by molecular cloning.

A transgenic mouse line with multiple copies of the human insulin gene integrated into its genome has been established. The degree of hyperinsulinemia correlates with human gene copy numbers. The transgenic mice provide a model system for studies in regulation of insulin gene expression and the effects of chronic hyperinsulinemia on glucose homeostasis.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47005-16 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Studies of Insulin Receptors in Circulating Cells in Man

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

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 R. Arakaki Medical Staff Officer DB/NIDDK

COOPERATING UNITS (if any)

Wayne State University (G. Grunberger)

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section; Biochemistry and Molecular Pathology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

1.5

1.0

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The present work continues prior investigations of insulin receptors on circulating cells in patients with insulin resistance and diabetes mellitus. The effects of diet, fasting and treatment on receptor function are under investigation. Insulin receptors are evaluated for their ability to bind insulin and to act as tyrosine-specific protein kinases.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47007-13 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Antibodies to Receptors: Detection in Disease States and Use as Probes

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

P.I.: S.I. Taylor Section Chief DB/NIDDK
 Others: B. Marcus-Samuels Chemist DB/NIDDK J. Roth Sci. Director NIDDK
 A. Cama Visit. Fellow DB/NIDDK P. Gordon Director NIDDK
 A. Ota Visit. Fellow DB/NIDDK
 D. Accili Visit. Fellow DB/NIDDK
 F. Barbetti Visit. Fellow DB/NIDDK
 D. LeRoith Visit. Sci. DB/NIDDK

COOPERATING UNITS (if any)

George Washington University (D. Moller, R. Ratner)
 New York University (S. Selinger)
 Sloan-Kettering Institute (O. Rosen)

LAB/BRANCH

Diabetes Branch

SECTION

Biochemistry and Molecular Pathophysiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

4.0

3.0

1.0

CHECK APPROPRIATE BOX(ES)

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

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

Antibodies directed against the insulin receptor have played a central role in investigations of the insulin receptor structure and function. Initially, these antibodies were identified in the serum of patients with autoimmune forms of extreme insulin resistance or hypoglycemia. All of the anti-insulin receptor autoantibodies in the original studies shared the ability to inhibit insulin binding. More recently, however, we have identified a patient whose serum contained anti-receptor antibodies which immunoprecipitated the insulin receptor without inhibiting insulin binding.

In addition, based on the recently elucidated primary sequence of amino acids in the human insulin receptor, we have synthesized peptides corresponding to specific structural domains in the receptor. Rabbits have been immunized with these peptides in order to develop anti-receptor antibodies directed against specific sites in the receptor. The antibodies have been employed to define the functions of these structural domains. In addition, anti-receptor antibodies have been used to identify structural abnormalities in patients with insulin resistant diabetes mellitus.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Positron Emission Tomography in Patients with Diabetes Mellitus

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

P.I. J. Roth Director, DIR NIDDK
 Others: R. Eastman Clinical Director DB/NIDDK

COOPERATING UNITS (if any)

D. Bogardus, Phoenix, AZ
 K. Baker, R.N. CC, A. Cassibry, RN, CC
 G. Berg MSF Nuclear Medicine

LAB/BRANCH

Diabetes Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL MAN-YEARS.

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Cerebral glucose metabolism has been studied using positron emission tomography (PET) under euglycemic clamp conditions in Pima Indians with normal, impaired glucose tolerance, and non-insulin-dependent diabetes mellitus (NIDDM). Each volunteer has undergone 2 studies, one during euglycemia (glucose 100 mg per dl and euinsulinemia), and one under euglycemia and hyperinsulinemia (plasma insulin approximately 1000 μ U/ml). In each study the glucose clamp is maintained for a half hour prior to the start of the PET scan which is indicated by intravenous injection of fluorodeoxyglucose (FDG). The PET data is accumulated for 2 hours, during which the euglycemic glucose clamp is maintained.

Static PET images obtained 45 minutes after injection of fluorodeoxyglucose have been analyzed and the local rates of glucose utilization in 201 regions of the central nervous system determined. Preliminary evaluation of the data obtained during the 2 clamp studies indicate a marked decrease in cerebral accumulation of FDG during hyperinsulinemia. This appears to be due to increased peripheral glucose uptake rather than due to depression of cerebral glucose metabolism. No specific area of altered glucose metabolism during hyperinsulinemia vs. euinsulinemia has been identified although scattered areas have shown significant alterations using uncorrected student T tests. The number of patients studied to-date is too small to allow firm conclusions to be reached regarding the effects of insulin on cerebral glucose utilization or to allow comparison of normal vs. diabetic Pimas and Pima vs. Caucasian controls.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47014-19 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Acromegaly and Growth Hormones

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

P.I.	P. Gorden	Section Chief	DB, NIDDK
Others:	C. M. Hendricks	Biol. Lab. Tech.	DB, NIDDK
	R. F. Arakaki	Senior Staff Fellow	DB, NIDDK

COOPERATING UNITS (if any)

none

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.0

OTHER:

.5

CHECK APPROPRIATE BOX(ES)

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

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

Acromegalic patients have continued to be followed with respect to pituitary irradiation. Further, we are evaluating the effects of transsphenoidal hypophysectomy in these patients and comparing them to the pituitary-irradiated patients.

A group of patients in a long-term follow-up study was evaluated to determine the effect of joint disease as a function of time following pituitary radiation. It appears that the joint disease is a function of the age of the patient and to the degree of involvement of initial therapy. Thus in patients with relatively severe joint disease, the joint disease progresses in spite of very significant reductions in growth hormone that occur following radiation therapy. Other studies underway are attempting to determine the effect of pituitary radiation on possible brain function or other complications of therapeutic maneuver.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47018 11 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cellular Hormone-Like Peptides

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

P.I.	J. Roth	Chief	DB/NIDDK
	D. LeRoith	Section Chief	DB/NIDDK
Others:	A. Liotta	Expert	DB/NIDDK
	Z. Yaniv	Guest Researcher	DB/NIDDK
	M. McKenzie	Staff Fellow	DB/NIDDK
	C.T. Roberts	Expert	DB/NIDDK
	W.L. Lowe	Med. Staff Fellow	DB/NIDDK
	L. Sankaran	Chemist	DB/NIDDK

COOPERATING UNITS (if any)

Laboratory of Cellular and Development Biology, NIDDK (J. Shiloach)

LAB/BRANCH

Diabetes Branch

SECTION

Molecular and Cellular Physiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

7.0

PROFESSIONAL:

7.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Substances similar to insulin, ACTH and somatostatin are present in unicellular organisms and higher plants. The studies have been extended to further characterize the insulin-ACTH- and somatostatin-related molecules in spinach, E.coli and Saccharomyces. Using gel chromatography and high performance liquid chromatography the extracted materials have been purified in preparation for amino acid sequencing.

To isolate the genes encoding these peptide hormones in multicellular non-vertebrates and unicellular organisms recombinant DNA technology is being used. Rat insulin-like growth factor I cDNA was cloned and sequenced to be used as an additional tool in the search for insulin-related genes in primitive eukaryotes and prokaryotes. Another use of this probe has been to study the regulation of IGF-I gene in rat. Differential splicing of primary transcript of rat IGF-I gene produces IGF-I mRNAs with at least three different 5'-untranslated region sequences. The relative steady state levels are differentially regulated in vivo by growth hormone in tissue-specific manner.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47019-11 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Morphologic Studies of Ligand Binding to Cells

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

P.I. P. Gorden

Section Chief

DB, NIDDK

COOPERATING UNITS (if any)

Institute of Histology and Embryology, University of Geneva
 School of Medicine, Geneva, Switzerland. (J.L. Carpentier,
 A. Roberts, L. Orci) - Foreign

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOXES)

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

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

This work represents over 11 years of collaboration between the Diabetes Branch and the Institute of Histology and Embryology at the University of Geneva. The initial observations demonstrated that polypeptide hormones are taken up by the cell through a process of receptor-mediated endocytosis similar to other biologically important ligands that bind to cells. In the present study, using election microscopy, we find a) there is an anatomical correlation between the dissociation of ^{125}I -insulin and its localization on the cell surface. This work has now been extended to include an insulin resistant cell line that has an abnormal surface which leads to a higher association of ligand to the non-villous portion of the cell surface. Further, receptor-mediated endocytosis also appears to be regulated in hypoinsulinemic states. In both rat and in human type I diabetes there is an inhibition of ^{125}I -insulin internalization in the hyperglycemic state, the normal state is restored by insulin treatment. The role of intracellular calcium on the endocytotic process as well as the relationship of stimulators of protein kinase C to internalization of both insulin and unrelated ligands such as transferrin have been studied also. In addition, the function of the small non-coated invaginations in receptor-mediated endocytosis are being investigated.

Formerly Z01 AM 47019-08 DB

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47021-10 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Cultured Cell Model for Hormone Receptors

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

P.I. P. Gorden

Director

NIDDK

COOPERATING UNITS (if any)

none

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

Project has been discontinued this year.

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Insulin Receptors in Syndromes of Extreme Insulin Resistance

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

P.I.:	S.I. Taylor	Section Chief	DB/NIDDK			
Others:	C. McKeon	Sen. Staff Fell.	DB/NIDDK	P. Gorden	Director	NIDDK
	K. Ojamaa	Visit. Fellow	DB/NIDDK	T. Kadowaki	Visit. Fellow	DB/NIDDK
	B. Samuels	Chemist	DB/NIDDK	C. Frapier	Guest Worker	DB/NIDDK
	V. Moncada	Guest Worker	DB/NIDDK	C. Hendricks	Biotech.	DB/NIDDK
	A. Cama	Visit. Fellow	DB/NIDDK	L. Beitz	HHMI	DB/NIDDK
	D. Accili	Visit. Fellow	DB/NIDDK	H. Kadowaki	Visit. Fellow	DB/NIDDK

COOPERATING UNITS (if any)

Whitehead Institute, Massachusetts Institute of Technology (Eric Lander)
Genentech, South San Francisco, CA (Axel Ullrich)

LAB/BRANCH

Diabetes Branch

SECTION

Biochemistry and Molecular Pathophysiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL

OTHER

8.6

5.6

3.0

CHECK APPROPRIATE BOX(ES)

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

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

Insulin resistance contributes to the pathogenesis of several human diseases such as obesity and non-insulin-dependent diabetes mellitus. We have investigated patients with genetic forms of extreme insulin resistance to gain insight into biochemical defects which give rise to disease.

Thus far, we have identified and characterized three mutant alleles of the insulin receptor gene. One insulin resistant patient (leprechaun/Ark-1) is a compound heterozygote who has inherited two alleles with distinct mutations. One allele has a missense mutation causing the substitution of glutamic acid for lysine at position 460 in the alpha-subunit of the receptor. This mutation increases the affinity of the receptor to bind insulin, and decreases the ability of acid pH to dissociate insulin from its receptor. The second allele has a nonsense mutation in which codon-672 is converted to a chain termination codon. This truncated receptor lacks the C-terminal, 48 amino acids of the alpha-subunit and the entire beta-subunit. Furthermore, the truncated receptor appears to be degraded rapidly and is not expressed at the cell surface. We have also identified two insulin resistant sisters from a consanguineous family. Both sisters are homozygous for a mutation causing the substitution of valine for phenylalanine in the alpha-subunit. This mutation is associated with impaired transport of the receptor to the plasma membrane.

An additional mechanism of insulin resistance is associated with a decrease in the levels of insulin receptor mRNA causing a decrease in the number of insulin receptors. We are currently cloning and characterizing the regulatory regions of the insulin receptor genes from these patients.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47024-09 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Biosynthetic Labeling of the Insulin Receptor

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

P.I.	R.F. Arakaki	Senior Staff Fellow	DB, NIDDK
Others:	E. Collier	Senior Staff Fellow	DB, NIDDK
	D.G. Rouiller	Visiting Associate	DB, NIDDK
	P. Gorden	Director	NIDDK

COOPERATING UNITS (if any)

none

LAB/BRANCH

Diabetes Branch

SECTION

Clinical Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.5

PROFESSIONAL:

3.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

We have studied the biosynthesis of the insulin receptor in human IM-9 lymphocytes. The alpha (135,000) and beta (95,000) subunits of the receptor are synthesized in the endoplasmic reticulum as a single M_r 190,000 glycoprotein with only high mannose oligosaccharide chains, i.e. proreceptor. This proreceptor is then transported to the Golgi complex where it undergoes proteolytic cleavage and carbohydrate processing. Direct analysis by high performance liquid chromatography of the carbohydrate chains of the insulin proreceptor demonstrate that the largest oligosaccharide found in control cells is $GLC_1MAN_9GLCNAC_2$ which represents only a small fraction (3%) of the total. The predominant proreceptor oligosaccharides are $MAN_9GLCNAC_2$ (25%) and $MAN_8GLCNAC_2$ (48%). Since a $GLC_3MAN_9GLNAC_2$ species is transferred cotranslationally, carbohydrate processing of the proreceptor is very rapid and limited to removal of the three glucoses and one mannose. Furthermore, in the presence of glucosidase inhibitors, castanospermine and 1-deoxynojirimycin, an abnormal precursor of M_r 205,000 is synthesized. The processing of this precursor to mature subunits is delayed and there is a reduction in cell surface insulin receptors. Thus, glucose removal is an important signal for processing of the insulin receptor. Additionally, we have found that the insulin receptor contains covalently linked fatty acids. Both the alpha and the beta subunits incorporate [3H]myristic and [3H]palmitic acids. The incorporation of fatty acid is dependent on protein synthesis and is found in the M_r 190,000 precursor. Thus, fatty acylation is a newly identified post-translational modification of the insulin receptor.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47025-05 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Tissue Receptors for Insulin and Insulin-like Growth Factors

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

P.I.	M. A. Lesniak	Chemist	DB/NIDDK
	F. De Pablo	Visiting Scientist	DB/NIDDK
	J. Roth	Director, DIR	NIDDK
Others:	M. Rojeski	Guest Researcher	DB/NIDDK
	F. Barbetti	Visiting Associate	DB/NIDDK
	N. Raben	Visiting Associate	DB/NIDDK

COOPERATING UNITS (if any)

Barcelona (Bassas, Girbau), U.S.-Spain

LAB/BRANCH

Diabetes Branch

SECTION

Receptor and Hormone Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

4.0

PROFESSIONAL:

4.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Insulin receptor and insulin-like growth factors (I and II) receptors structure studies have been extended in rat brain. The binding of labeled peptides to thin sections of frozen fresh rat brain was visualized with autoradiography. By several criteria including structure-activity relationship analysis, these brain peptides receptors were qualitatively indistinguishable from peptide receptors previously characterized on brain and other more typical target tissues and distinct from each other. Each peptide exhibits its own distinctive binding pattern, i.e., each peptide binds to specific cytoarchitectonic structures.

Chicken embryos are a suitable model for studying the role of insulin, IGF-I and IGF II and their receptors in embryogenesis. Multiple chick embryo tissues exhibit insulin and IGF-I binding. We have studied the tissue-specific structure differences and the developmental regulation in brain, liver, muscle, heart and limb buds. We have demonstrated that the insulin receptors are active since anti-insulin receptor antibodies cause morphological and biochemical perturbation of development.

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Tyrosine-Specific Protein Kinase Activity Associated with the Insulin Receptor

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

P.I.: S.I. Taylor Section Chief DB/NIDDK
 Other: R.N. Margolis, I.P.A., Howard University, Washington, D.C.

COOPERATING UNITS (if any)

East Carolina University (Jose F. Caro)
 Columbia University College of Phys. & Surgns. (Dr. Robert Rees-Jones)
 Howard University, Washington, D.C. (D. Seminara, R. Margolis)
 Johns Hopkins (A. Hubbard)

LAB/BRANCH

Diabetes Branch

SECTION

Biochemistry and Molecular Pathophysiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

In first step of insulin action, insulin binds to its receptor on the surface of the target cell. The insulin receptor is a transmembrane protein which possesses a tyrosine-specific protein kinase. When insulin binds to the extracellular domain of the receptor, this activates the receptor's tyrosine kinase. A growing body of evidence suggests that the activation of the tyrosine kinase is a necessary step in initiating the biological actions of insulin. Accordingly, we have embarked upon a search for intracellular proteins which are substrates for phosphorylation by the receptor-associated tyrosine kinase. We have identified one such substrate in rat liver plasma membranes: a glycoprotein with an apparent molecular weight of 120,000 daltons (pp120). pp120 is present in liver from several species, but has not been identified in other tissues.

We have demonstrated that pp120 is identical to HA4, a glycoprotein localized to the bile canalicular domain of the hepatocyte plasma membrane. Monoclonal antibodies to HA4 have been used to immunoaffinity purify HA4/pp120. We are obtaining partial amino acid sequences of purified HA4/pp120. This amino acid sequence data will be used to clone cDNA encoding HA4/pp120.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47027-03 DB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Use of SMS 201-995 in Hormone Secreting Tumors

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

P.I.	P. Gorden	Director	NIDDK
Others:	R. J. Comi	Senior Staff Fellow	DB, NIDDK
	R. F. Arakaki	Senior Staff Fellow	DB, NIDDK
	B. Weintraub	Chief	MCNEB, NIDDK
	N. Gesundheit	Senior Staff Fellow	MCNEB, NIDDK

COOPERATING UNITS (if any)

none

LAB/BRANCH

Diabetes Branch

SECTION

Clinical and Cellular Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

We have studied the use of the long-acting somatostatin analogue, SMS 201-995, in patients with acromegaly, TSH secreting pituitary tumors and glucagonomas. These studies have defined 1) an appropriate dose and schedule for control of TSH secreting pituitary adenoma and its resultant hyperthyroidism, 2) an appropriate subgroup of acromegalic patients in whom this analogue, given thrice daily, controls GH hypersecretion: 3) the effects of the drug in glucagonoma syndrome in terms of control of glucagon hypersecretion and correction of hypoaminoacidemia. Our current studies have focused on the long term use of this agent in acromegaly and patients with TSH secreting tumors and the correlation of hormonal effects with symptomatic benefit. In addition, our studies indicate that all patients develop thickened bile accumulation in the gallbladder while receiving treatment, which may progress to gallstones.

ANNUAL REPORT OF THE CLINICAL HEMATOLOGY BRANCH

National Institute of Diabetes and Digestive and Kidney Diseases

- I. Study of Immunology of Blood Cell Deficiencies
- A. Identification of Platelet Proteins Reactive with Normal Serum Immunoglobulins on Immunoblots. Characterization of a 95 KD Ig Receptor

In the past 3 years several investigators have identified apparent autoantibodies using the Western blot (WB) technique in sera of patients with post transfusion purpura, drug purpura, sepsis, AIDS, SLE, and ITP. However, our studies suggest that some WB reactions interpreted by others as evidence of autoimmunity or as a specific for certain surface platelet antigens, namely GPIIIa, IV, or V, may simply be due to quantitative variations in normally occurring immunoglobulins. Many normal sera, when incubated with Western blots (WBs) of normal whole platelets, produce a 95KD band upon addition of anti-IgG -IgA, or -IgM, the frequencies being 65%, 50%, and 30%, respectively. This band occurs in addition to "background" bands which are produced by antiglobulin exposure alone and which represent intact or fragmented platelet-associated Igs and/or platelet proteins which share Ig epitopes. Rarely, normal sera also produce other bands at 100-110, 80-85, 60-75 and 50-60 KD. Titers of 95 kD-reactive IgG in normal sera are in the range of 10-1280 with 85% between 10 and 50; and, whether high or low, remain stable over at least several years. Commercial IgGs have titers equivalent to normal serum titers of 50 to 130. Purified normal F(ab')₂ reacts at similar molarity to parent IgG. The 95 kD reaction occurs with autologous or homologous sera and equally with all normal platelets, but not with WBCs or RBCs. The 95 kD protein differs from glycoproteins (GPs) IIIa, IV, and V because: (1) Thrombasthenic, Bernard-Soulier, and normal platelets react equivalently. (2) Intact platelets do not absorb the 95 kD-reactive IgG and give negative FACS reactions, indicating that the receptor is not exposed on the platelet surface. (3) Approx. 90% of the 95 kD protein is recoverable in the ultrafiltrate of sonicated, freeze-thawed platelets. (4) Under reducing conditions and after thrombin treatment, its Mr and immunologic reactivity are unchanged. (5) It does not stain with PAS and is not selectively bound by Concanavalin A. (6) Eighty-seven percent of sera from thrombocytopenic patients (10 ITP, 5 drug purpura, 5 SLE, 2 HIV infection, 1 myelodysplasia) reacted with the 95 kD protein, titering from 10 to 2000 (mean 190) with anti-IgG; but none of these sera reacted with GPIIIa, IV, or V. Patterns with sera from thrombocytopenic patients duplicate those of normal sera but reaction titers tend to be higher. The 95 kD reaction may have a role in platelet homeostasis and under certain pathologic conditions could be responsible for increases in platelet-associated Igs. Purification of the internal 95 kD Ig receptor protein has been undertaken recently. Starting material consisting of dialyzed, filtered platelet lysate supernatant, was subjected first to anion exchange (Fast Q) chromatography, which effected separation the 95 kD protein from GPIIIa, and then to molecular

sizing and chromatofocusing. Preliminary data suggest the pI of this protein is in the 5-6 range. When possible, analysis of the fully purified protein and search for amino acid sequence homologies to other known proteins may help explain the presence of naturally occurring 95 kD-reactive-antibodies and their possible role in platelet turnover.

B. Pathophysiology, Treatment and Serology of Post-Transfusion Purpura

Post transfusion purpura (PTP) (Shulman's disease) is a disease caused by a mismatched platelet transfusion in which antibodies appear against the foreign transfused platelet antigen and the sensitized patient develops thrombocytopenia. When we described the disease in 1959 we proposed that adsorption of antigen-antibody complexes from soluble antigen of transfused platelets were responsible for thrombocytopenia. Until recently immunologic techniques have not been sensitive enough to evaluate this theory. We have studied 4 cases of PTP to determine whether we can detect circulating immune complexes. We have found that platelet antigens that cause PTP circulate free from platelets in donor plasma (approximately 0.7% of the circulating platelet surface antigen content) and can be adsorbed by platelets lacking the antigen. Both allelic forms (PlA¹ & PlA²) can be adsorbed in this way. Platelet membrane preparations are also capable of adsorbing these alloantigens. Our antigen detection system involves in vitro adsorption of free antigen from centrifuged and filtered platelet poor plasma (PPP) onto platelets lacking the antigen, followed by SDS/PAGE and western transblot (WB) analysis. The transblotted antigen is incubated in the presence of dilute antisera (or a purified antibody eluate) from PTP plasma and visualized by a reaction with an enzyme-linked antihuman antibody reagent. Using this method in titration experiments, we are able to detect as few as 1×10^6 whole platelets, equivalent to 5×10^{10} antigen molecules (approximately 8×10^{-14} mole of antigen). Quantitation of free antigen in apheresis plasma from four PTP patients yielded values less than 5×10^{10} antigen molecules per 80 ml of PTP plasma (less than 6.25×10^8 antigen molecules/ml). These low values are in all likelihood due to removal of transfused antigen by reactions with antibodies in circulation. Only 200-400 Ag-Ab complexes per platelet are sufficient to cause platelet destruction. At these values, destruction of all circulating platelets requires $1.45 - 2.9 \times 10^{14}$ circulating free antigen molecules, or a concentration of $5-10 \times 10^{10}$ antigen molecules/ml of plasma. Since this is within the range of our present detection system it leaves open the opportunity for the detection and quantitation of the foreign platelet antigen in patients transfused with mismatched platelets.

C. Serology of Platelet-Bound Antibodies and Anticardiolipin (ACL) Antibodies in Patients with Systemic Lupus Erythematosus

A subset of patients with SLE develop unexplained thrombocytopenia. In addition, many SLE patients have measurable serum titers of ACL antibodies. Because ACL antibodies have been found to cross react with platelet phospholipid, it is of interest to determine whether presence of these antibodies correlates with thrombocytopenia. In cooperation with MD:NIDDK and AR:NIAMS we developed a method employing a fluorescence-activated cell sorter (FACS) for quantitation of antiplatelet antibodies directed against platelet surface antigens, using sera containing well-characterized platelet alloantibodies and drug-dependent antibodies. This method was then applied to study SLE sera which had been previously tested by EIA for ACL antibodies. Of approximately 30 patients, 50% had IgG antibodies that bound to subpopulations of normal platelets. However, the presence of these antibodies did not correlate with titer of ACL antibodies or with platelet count.

D. International Study of Neonatal Thrombocytopenia

CHB-NIDDK is one of the labs participating in an international study of Neonatal Thrombocytopenia conducted by Dr. James Bussel at Cornell Medical Center, New York. Blood samples, usually from local hospitals are submitted to our lab periodically for work-up of neonatal thrombocytopenia which includes phenotyping of the parents' and, if necessary, the baby's platelets and screening the mother's serum for anti-platelet antibodies. In addition, when there is a history of maternal ITP, quantifications of Platelet-associated IgG (PAIgG) are performed. Patients are assigned a number to maintain anonymity and pertinent lab results are forwarded to Dr. Bussel. One of his assistants then contacts the local physician for a complete birth history and details of treatment, if any, given for the thrombocytopenia. Information characterizing the mechanism of TP, aids in decision making for management of the infant and in the specific instance of isoimmune TP can alert the physician to monitor future pregnancies more closely. Fifty three cases accumulated so far have provided information on the value of treating mothers antenatally with adrenocorticosteroids that cross the placenta and with higher doses of I.V. IgG to prevent fetal hemorrhage at birth.

E. Development of a Microtiter EIA for Platelet Phenotyping

An important function of a regional Blood Bank(BB) is to supply specially typed platelets when needed for transfusion to patients with post-transfusion purpura and to infants with isoimmune neonatal thrombocytopenia. Until recently large scale typing of BB donors for the PLAI antigen has been difficult due to the labor-intensive assays used, including complement fixation and Western blotting. We screened 100 donors in this manner in 1986 and identified 3 with PLAI-negative phenotypes. This year, in collaboration with WGMCC:TM , an EIA microtiter plate assay was

developed for PlA1 phenotyping. Approximately 500 donors were screened over 6 mos. and 12 of these were PlA1-negative. Each was confirmed PlA1-negative by the conventional techniques. This screening assay can be retailored to phenotype platelets for other antigens, namely Lek/Bak, Pen/Yuk, and PlA2, which less commonly account for disorders of allosensitization and for which we have well-characterized anti-sera derived from patients referred to us with alloimmune platelet disorders.

II. Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

A. A New Mechanism of Platelet Activation By Small Cross-Linking Reagents

In studying the effects of sulfonated stilbene anion channel blockers (ACB) on platelets we noticed that the most potent analog, 4,4'-diisothiocyano-2,2' disulfonic acid stilbene (DIDS), caused platelets to secrete and to aggregate. Platelet responses to DIDS are equivalent in rate and intensity to those caused by thrombin or A23187 and similar in not requiring extracellular Ca^{++} or fibrinogen. The Kd for activation by DIDS is $5 \times 10^{-5}M$ with a maximum at $10^{-4}M$. DIDS activation is decreased by indomethacin ($100 \mu M$) and apyrase (1 mg/ml) and totally blocked by PGE_1 ($10 \mu M$). Platelet activation by DIDS appears to be caused by specific close cross-linking of membrane surface proteins and not by the inhibition of anion channels because: 1) DIDS can covalently bind protein amino groups via its isothiocyano termini and cross-link a span of 12.7 \AA . 2) The ACB, SITS, which is a monovalent isothiocyano form of DIDS, does not activate platelets but competitively inhibits their response to DIDS by saturating potential cross-linking sites. 3) Increasing concentrations of DIDS between $10^{-4} M$ and $10^{-3} M$ decreases platelet activation to 10% of maximum by similar competition. 4) Another cross-linking agent, 3,3'-Dithiobis(sulfosuccinimidylpropionate) (DTSSP), which spans 12 \AA and has amino group specificity, also activates platelets but with lower affinity and lower intrinsic activity than DIDS. 5) Large lectins which weakly cross-link membrane glycoproteins (GP) cause only a slight and delayed platelet activation, and agents that nonselectively cross-link membrane proteins, like formaldehyde, do not promote secretion. 6) Platelet membrane proteins with molecular weights of 52, 170 and 230 kD were specifically labelled by saturating surface protein amino sites with excess DIDS, reacting the free isothiocyano group of DIDS with C^{14} -methylamine, and then performing SDS-PAGE and autoradiography. GP IIB/IIIa is not involved because DIDS activates thrombasthenic platelets. This 12.7 \AA inter- and/or intra- molecular cross-linkage, possibly involving GPIb (based on M_r), may help identify structure-activity relationships of other agonists and clarify the mechanisms of membrane signal transduction.

B. Suramin Clinical Study

In studying the effects of polysulfonated aromatic anion channel inhibitors like suramin, on platelets we noticed that: 1) When added to platelet rich plasma, Suramin, greater than $10^{-4} M$ causes platelet aggregation, and 2) Suramin, at $10^{-5} M$, potentiates the effective thrombin activity 2-fold above controls. We wondered if these activities would be realized clinically when suramin is used in the treatment of parasitic infections or in its newer application as an antiviral agent in the treatment of AIDS and as an oncolytic agent in the treatment of adrenal carcinoma. In collaboration with the

Clinical Pharmacology Branch, NCI, we studied clotting and platelet function in patients undergoing suramin treatment for end stage adrenal carcinoma. In vivo, suramin is highly protein-bound without significant metabolism and has a very long half life. Initial clinical studies employed 2.2 gm I.V. doses infused over 60 minutes. Thrombin times, which reflect the ability of thrombin to clot plasma fibrinogen, showed a biphasic change with a decrease in effective thrombin concentration to 30 % of control at 30 minutes, a potentiation to 120 % of control at 60 minutes and return to control values at 120 minutes. The platelet count was also affected by suramin administration, decreasing during acute infusions but returning to normal within hours. These effects were correlated with plasma suramin concentrations during and after the loading dose infusion. Patients given 20 mg/kg suramin/24 hours for four days did not experience the same thrombin times alterations as described above, but their platelets increased in sensitivity to various agonists. During infusion platelets showed an increase in aggregability to epinephrine (33 fold), ADP (4 fold) and collagen (2000 fold). The increased sensitivity was still observed two to three weeks after the initial suramin exposure. Increased bleeding tendencies with increased prothrombin time and low platelets experienced by patients on suramin appear to reflect this drug's ability to alter thrombin clotting activity, change platelet distribution and affect platelet sensitivity.

C. Study of Platelet Hyperaggregability and Thrombin-Induced Malondialdehyde Generation in Patients with Microvascular Angina

Platelet "hyperaggregability" with resultant microvascular occlusion has been put forth as one possible explanation for chest pain in patients with angiographically normal coronary arteries. In addition, platelets in normals have been found to be hyperaggregable between the early morning hours of 6 and 9 a.m., the same hours during which there is a statistically higher frequency of myocardial infarction and sudden cardiac death. Investigators in CB:NHLBI have recently described a subset of patients with chest pain and angiographically normal coronaries who have impaired coronary flow reserve in response to pacing and increased coronary resistance post ergonovine, suggesting a microvascular abnormality. In addition, many of these patients have abnormal esophageal motility and abnormal forearm vasodilator responses, raising the possibility of a generalized smooth muscle abnormality. In cooperation with CB:NHLBI, we studied platelets of cardiac patients to determine whether they aggregate differently than normals in response to standard agonists (ADP, and epinephrine). Malondialdehyde (MDA) generation by platelets in response to thrombin-stimulation was also studied as a more quantitative reflection of platelet activation. Fifteen tests were performed on a total of 13 patients, 9 with microvascular angina and 4 with hyperthrophic cardiomyopathy. Results can be summarized as follows: (1) There is a wide range of platelet sensitivity to ADP and epinephrine among normal controls (threshold doses for aggregation ranged from 0.5 to 20 μ M ADP and 0.25 to 10 μ M epinephrine). (2) No consistent differences in platelet aggregability or MDA production were observed between microvascular angina patients

and controls. Our data did not corroborate the findings of others. Furthermore, use of in vitro tests such as these which require anticoagulation and mechanical separation of platelets from whole blood cannot be expected to accurately reflect in vivo conditions which may predispose to development of platelet microaggregates.

D. Studies of Congenital Microthrombocytopenias

We have studied two male unrelated children, aged 3 and 8 yrs, who have congenital microthrombocytopenias which have predisposed them to cutaneous purpura and occasional life-threatening hemorrhages. Neither child fulfills the usual criteria for Wiskott-Aldrich syndrome (W-AS), an X-linked recessive disorder which consists of microthrombocytopenia, cellular and humoral immunodeficiencies and eczema. Concise diagnosis is desirable both for management and family counselling. W-AS is the only disease associated with microthrombocytopenia in males. Tests performed thus far have included: (1) Homologous ¹¹¹In-labeled platelet survivals done at times when platelet transfusions were indicated for treatment of hemorrhage. In each case homologous platelets survived normally. (2) An autologous survival done in the older child was shortened in proportion to the depression in platelet count. (3) Immunologic work-ups including quantitative serum immunoglobulins and isohemagglutinins were all in normal range for age which differs from W-AS. (4) Electron microscopic analysis of glutaraldehyde-fixed platelets in one child were normal with the exception of size. (5) SDS-PAGE analysis of solubilized platelets indicated normal major glycoprotein patterns rather than deficient GP Ib or Ia as has been reported for W-AS. These patients appear to represent an, as yet, undescribed syndrome different from W-AS.

E. Effects of Newly Recognized Mucosal Peptide Antimicrobials On Platelets

Small mucosal peptides have been shown to be part of antimicrobial defense mechanisms in amphibians and mammals, and similar compounds are also found in insects. One such human protein, uteroglobulin, is secreted by the uterus in response to embryo implantation. Recently this protein was found to inhibit thrombin-induced platelet aggregation and we have studied the mechanism of this inhibition. Dr. Anil Mukherjee has synthesized small peptide fragments of uteroglobulin in search of its active site. They have found that some of these peptides are potent inhibitors of phospholipase A₂, a membrane enzyme that releases arachidonic acid and is tied to receptor-mediated signal transduction. We have used these peptides in experiments with thrombin, ADP, collagen and calcium ionophore-stimulated platelet aggregation and secretion. The whole molecule is not effective in inhibiting aggregation or thrombin enzymatic activity but a small four amino acid fragment has potent ability to block thrombin, ADP and A23187-induced aggregation. It did not, however, inhibit platelet secretion of serotonin which is the initial step of platelet aggregation. This suggests blockade occurs in the steps after receptor-mediated cellular activation. The most likely mechanism of action is blockade of fibrinogen binding to platelets after fibrinogen receptors are exposed by activation of the

cell. Fibrinogen recognizes its receptor through a four amino acid sequence, RGDS, common to most other attachment proteins like fibronectin or vitronectin. The anti-inflammatory, antibacterial peptides therefore appear to prevent local platelet aggregation without affecting other platelet activity. The intact uteroglobulin molecule may be hydrolyzed by local tissue or neutrophil enzymes to release the small peptides responsible for this specific action. Currently we are screening amphibian mairanins and insect peptides (cercopins) for similar types of inhibitory activity to see if this general mechanism has been conserved through development.

F. Platelet Survival In ITP

On the basis of data from autologous platelet survival studies, a number of investigators have recently concluded that reduced marrow platelet production explains depressed platelet counts in 30% of patients with ITP. Because our previous studies of ITP indicated shortening of survival approximately proportional to platelet count in all cases, we looked at differences in survival techniques as a possible explanation for the new conclusions. Until recently platelet survivals in ITP were measured with homologous ^{51}Cr platelets. The new $^{111}\text{Indium}$ labelling technique permits use of autologous platelets but presents difficulties in obtaining platelets from thrombocytopenic patients. Often platelets from thrombocytopenic blood cannot be fully separated from red cells (RBCs). Survivals in recent ^{111}In studies may have been overestimated because RBC contamination of the labelled platelet infusion was overlooked. RBCs are also labelled by ^{111}In and circulate approximately 10 times longer than normal platelets. When we performed extra centrifugations to reduce RBC content of platelet preparations from thrombocytopenic blood, ^{111}In survival values became appropriately shortened. Parallel homologous platelet survival studies done on the same patients supported this finding. We conclude that platelet destruction, not reduced production, can fully explain low platelet counts in ITP. Furthermore, data on the rates of rise of platelet counts in response to corticosteroids or high dose IV IgG given to chronic ITP patients indicate that production, if altered, is increased over normal.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 51,000-30 CHB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Study of Immunology of Blood Cell Deficiencies

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

PI:	N.R. Shulman	Chief	CHB, NIDDK
Others:	D.M. Reid	Senior Staff Fellow	CHB, NIDDK
	J. Vostal	Research Fellow	CHB, NIDDK
	C.E. Jones	Chemist	CHB, NIDDK

COOPERATING UNITS (if any)

J. Balow, S.E. Pillemer, M. Cronin (NIDDK); J. Hoofnagle (NIDDK); E. Reed, C. Carter (Blood Bank); T. Bussell, N.Y. Hospital; T.J. Kunicki (Blood Center) Milwaukee; C. Leisinger, (Tulane Univ.)

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2

PROFESSIONAL

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

Idiopathic thrombocytopenic purpura (ITP), neonatal purpura (INT) post-transfusion purpura (PTP), drug-purpura and thrombocytopenia associated with infection or altered immune states are the major immunologic thrombocytopenias. Antibody reactions in these disorders are relevant to autoimmunity, histocompatibility, malignant surveillance, alloimmunity, pathogenicity of antigen-antibody complexes and cellular immune injury generally. We have identified internal platelet proteins that bind immunoglobulins by Western blot (WB) and have purified and characterized a 95 kD receptor by anion exchange chromatography and HPLC molecular sieving and chromatofocusing. Platelet alloantigens responsible for post transfusion purpura were found to circulate in normal plasma and to be adsorbed in sufficient amounts as antigen-antibody complexes to account for thrombocytopenia in transfused subjects. Anti-cardiolipins (ACLs) have been found on platelets of lupus erythematosus (SLE) patients but do not correlate with serum ACLs or with platelet counts. Although 30% of 90 SLE patients had elevated platelet-associated IgG (PAIgG) and other positive serologic tests for presumed anti-platelet antibodies their autologous platelet survivals with III-In were normal. On the basis of 53 cases of alloimmune neonatal thrombocytopenia the value was established of treating mothers antenatally with adrenocortico steroids and I.V. IgG that cross the placenta to prevent fetal hemorrhage at birth. A microtiter EIA was developed to facilitate phenotyping platelet antigens for matching transfusions in a Blood Bank setting.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 51,001-30 CHB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

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

PI: N.R. Shulman Chief CHB:NIDDK

Others: Diane M. Reid Senior Staff Fellow CHB:NIDDK
J. Vostal Research Fellow CHB:NIDDK
Charles E. Jones Chemist CHB:NIDDK

COOPERATING UNITS (if any)

S. Epstein, R. Cannon (NHLBI); M. Zaslov, Univ. of Penn.; A.B. Mukherjee (NICHD); T. Lawley, R.A. Swerlick (NCI), Emory Univ.; C.E. Myers, C. Stein (NCI)

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2

PROFESSIONAL:

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

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

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

The physiology of platelet secretion has many features in common with the secretory physiology of endocrine and neuronal cells; and a number of the biogenic amines synthesized, stored, and secreted by these different cell types are similar. Platelet membrane glycoproteins (GP) appear to be major factors determining cell-cell recognition, adhesion and secretion. We have discovered a new mechanism of platelet activation by small cross-linking reagents that is independent of Ca^{+2} and fibrinogen. The most potent compound, a disulfonic acid stilbene (DIDS), with a 12 Å span, reacts with platelet membrane proteins of molecular weights 52, 170, and 230 kD, possibly involving glycoprotein Ib, a major adhesion protein of platelets. Suramin, a drug used in experimental treatment of AIDS and malignancies was found to inhibit the active site of thrombin, to inhibit thrombin-induced platelet activation, and to cause variations in the thrombin time and, increases in platelet aggregability as much as 20-fold normal. In patients with microvascular angina, we found no evidence to support abnormal platelet responsiveness to standard agonists. Two patients with congenital microthrombocytopenia of a degree similar to that of Wiscott-Aldrich syndrome (WAS) were found to have shortened platelet survival but normal immunologic assessments, electron micrograph of platelets, and membrane glycoproteins. They appear to represent an, as yet, undescribed syndrome differing from WAS. Newly recognized mucosal peptide antimicrobials, maganins and uteroglobulin (UG) were found to inhibit platelet aggregation by preventing the fibrinogen-receptor interaction without affecting platelet secretion. One hydrolytic 4 peptide fraction of UG was most active, the intact UG least active. Platelet survival in ITP utilizing ^{111}In indicated that platelet destruction, not decreased production accounts for development of ITP.

ANNUAL REPORT OF THE GENETICS AND BIOCHEMISTRY BRANCH

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Biochemical Genetics Section

Dr. Proia and his colleagues have continued their studies of the lysosomal enzyme β -hexosaminidase (a deficiency of which is responsible for Tay-Sachs disease). They have characterized the glycosylation sites of this enzyme and have determined the effect of individual oligosaccharides on the catalytic activity, transport and processing of the enzyme. They have determined that four of the five potential glycosylation sites (Asn-X-Ser/Thr) of the hexosaminidase β -chain were modified by the addition of oligosaccharide chains. The lack of any one of the oligosaccharides did not dramatically affect catalytic activity or the delivery of the enzyme to lysosomes. They have also demonstrated that only two of the the four oligosaccharide chains were not phosphorylated. When these two phosphorylated sites were removed by mutagenesis, the enzyme was not delivered to lysosomes.

In a separate project a hexosaminidase α chain cDNA has been cloned from a library prepared with the fibroblast mRNA of a patient with the adult form of Tay-Sachs disease. They are currently sequencing the clone to identify the mutation responsible for this form of the disease.

Dr. Robbins and her collaborators have continued their studies to dissect the processes of endocytosis, glycoprotein biosynthesis and sorting. Their approach has been to isolate and analyze CHO mutants. They have shown that most CHO endocytosis mutants fall into two genetic complementation groups, End1 and End2; both classes of mutants are defective in endosomal acidification. They have identified a candidate for the End2 protein using three-dimensional gel procedure.

Molecular Genetics Section

Dr. Ackerman and collaborators have continued their work on their analysis of the mode of action of the Aspergillus toxin Alpha-sarcin and related toxins. Alpha-sarcin produces a precise cut near the 3' end of 28S ribosomal RNA in vitro only if the ribosomes are pretreated with puromycin and EDTA. Dr. Ackerman's group has now shown that this specific cleavage occurs in living Xenopus oocytes by microninjecting the toxin. Ricin, Shiga toxins, and shiga-like toxin variant appear to leave similar effects to alpha sarcin in oocytes.

On a second project Dr. Ackerman and his colleagues have shown that oocytes efficiently repair microinjected DNA containing pyrimidine dimers.

Dr. Camerini-Otero and his colleagues have continued their studies of genetic recombination in eukaryotes. They have reported the partial purification and characterization of a strand exchange protein or recombinase from nuclear extracts of human cells and tissues and embryos of *Drosophila melanogaster*. Recently they have used a variety of assays to show that both *E. coli* and human recombinase can form stable joint molecules from substrates that share only very small regions of homology (as little as 13 bp in one case). This finding has two important implications. First, these proteins can recognize and pair in vitro very short regions of homology. This result is consistent with data from both prokaryotic and eukaryotic systems that demonstrate that genetic recombination in vivo can utilize exceedingly short stretches of DNA homology. Second, the surprising stability, both to deproteinizing agents and to temperature, of joint molecules containing short hydrogen-bonded regions, suggests that these structures do not have a displaced strand that is free to participate in branch migration.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52008-09 GBB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Gene Expression and Human Genetics

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

PI:	R. D. Camerini-Otero	Chief	GBB, NIDDK
Others:	P. Hsieh	Senior Staff Fellow	GBB, NIDDK
	A. Eisen	Med. Staff Fellow	GBB, NIDDK
	C. S. Camerini-Otero	Med. Staff Fellow	GBB, NIDDK
	F. Mills	Guest Researcher	GBB, NIDDK
	R. Kiyama	Visiting Fellow	GBB, NIDDK
	R. Gardner	Med. Staff Fellow	GBB, NIDDK
	L. Milner	Bio. Lab. Tech.	GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

8.0

PROFESSIONAL:

6.5

OTHER:

1.5

CHECK APPROPRIATE BOX(ES)

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

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

Genetic recombination is a multistep process involving many gene products. In order to dissect the biochemical steps involved we have chosen to focus on a key early step: strand exchange between homologous parental DNAs. To date, the ability to carry out a strand exchange between a linear duplex DNA and a homologous circular single-strand DNA is unique to recombination proteins. The product of this strand exchange reaction is a joint molecule composed of a single-strand circle joined to one end of a linear duplex. Three proteins responsible for this step have been purified: *uvx*s from phage T4; Rec A from *E. coli*; and rec 1 from *U. maydis*.

Over the last two years we have reported the partial purification and characterization of similar strand-exchange proteins or recombinases from nuclear extracts of human cells and tissues and embryos of *D. melanogaster*. The proteins have two noteworthy characteristics: (1) they do not require ATP (unlike Rec A and rec 1); and (2) their direction of strand displacement (3' to 5') was similar to that of rec 1 but opposite to that of Rec A.

Recently, we have used a variety of assays to show that both *E. coli* Rec A and human recombinase can form stable joint molecules from substrates that share very small regions of homology (as little as 13 bp in one case). This finding has two important implications. First, these proteins can recognize and pair *in vitro* very short regions of homology. This result is consistent with data from both prokaryotic and eukaryotic systems that demonstrate that genetic recombination *in vivo* can utilize exceedingly short stretches of DNA homology. Second, the surprising stability, both to deproteinizing agents and to temperature, of joint molecules containing short hydrogen-bonded regions, suggests that these structures do not have a displaced strand that is free to participate in branch migration.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 52009-09 GBB

PERIOD COVERED

October 1, 1987 through December 31, 1987

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

Endocytosis, Secretion and Compartmentalization in Mutant CHO Cells

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

PI: A.R. Robbins Research Geneticist LBM, NIDDK

Others: C.W. Hall Research Chemist LBM, NIDDK
 S.M. Laurie Visiting Associate LBM, NIDDK
 C.F. Roff Senior Staff Fellow LBM, NIDDK

COOPERATING UNITS (if any)

Department of Biochemistry, School of Public Health, Johns Hopkins University
 (S.S. Krag)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

1.0

PROFESSIONAL:

1.0

OTHER

CHECK APPROPRIATE BOX(ES)

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

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

This project transferred to LBM. The new project number is Z01 DK 18009-09 LBM.

Our approach to dissecting the processes of endocytosis, glycoprotein biosynthesis and sorting is through isolation and analysis of mutants. We have previously shown that most CHO cell endocytosis mutants fall into two genetic complementation groups, End1 and End2; both classes of mutants are defective in endosomal, but not lysosomal, acidification. Having identified a candidate for the End2 protein, Calvin F. Roff has developed a novel preparative three-dimensional gel procedure for purification of this (and other) membrane proteins in quantities sufficient for immunization.

To obtain new classes of mutants we devised an isolation procedure for cells defective in lysosomal acidification. Exploiting the quenching of fluorescein at acidic pH we screened for cells exhibiting above normal fluorescence after pulse-chase labeling with fluoresceinated dextran (Mr 70,000). One such mutant accumulates dextran in large non-acidic vacuoles; based on functional assays, its endosomal acidification is unimpaired.

Susan M. Laurie has continued analysis of LEFIC, a mutant Ltk- cell which is cross-resistant to toxins but has normal endosomal function. The principal defect in LEFIC appears to involve movement of membrane proteins from late Golgi regions to the plasma membrane. Oddly, delivery of membrane proteins in LEFIC is more severely affected than is secretion of soluble proteins.

To further characterize mutants defective in early steps in the pathway of N-linked glycosylation, Clara W. Hall has developed an *in vitro* system for biosynthesis, translocation and elongation of lipid-linked oligosaccharides in intact microsomal vesicles. Conditions for measurement of translocation of lipid-linked Man5GlcNAc2 (the intermediate believed to move from external to luminal faces of the ER) without elongation of the oligosaccharide have been established.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 52011-04 GBB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Oocyte Specific Genes in Amphibian Embryogenesis

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

PI:	Eric Ackerman	Senior Staff Fellow	GBB, NIDDK
Others:	Shailendra K. Saxena	Visiting Fellow	GBB, NIDDK
	John Hays	Sabbatical Professor	GBB, NIDDK
	Jitendra K. Saxena	Visiting Fellow	GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.8

PROFESSIONAL:

2.5

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

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

I. The Aspergillus toxin alpha-sarcin produces a precise cut near the 3'-end of 28S ribosomal RNA in vitro only if the ribosomes are pre-treated with puromycin and EDTA. Alpha-sarcin can also behave as a general nuclease in vitro under appropriate conditions. In order to investigate alpha-sarcin's in vivo activity, we injected it into living Xenopus oocytes and analyzed the resulting RNA. We have also investigated whether ricin, Shiga toxin, and Shiga-like toxin variant (SLT-IIv) produce similar effects in oocytes.

II. During early development Xenopus replicates its DNA nearly as fast as E. coli in log phase; perhaps indicating that oocytes may be an excellent source of DNA repair activity. We have investigated pyrimidine-dimer repair by microinjecting uv-irradiated DNA into oocytes and assaying for repair using 2 methods: (1) Transformation of repair deficient E. coli mutants with the microinjected DNA; (2) Absence of pyrimidine dimers using UV-Endonuclease and denaturing agarose gels.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 52012-04 GBB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Structure-Function Relationships of Lysosomal Enzymes

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

PI:	Richard L. Proia	Senior Staff Fellow	GBB, NIDDK
Others:	Sybille Sonderfeld-Fresko	Guest Researcher	GBB, NIDDK
	Ruth Navon	Visiting Associate	GBB, NIDDK
	Gabrielle Weitz	Visiting Fellow	GBB, NIDDK
	Leon Eidels	Special Volunteer	GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.8

PROFESSIONAL:

2.5

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

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

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

I. We have characterized the glycosylation sites of human β -hexosaminidase B and have determined the effect of individual oligosaccharides on the catalytic activity, transport and processing of the enzyme. Glycosylation is an essential step for the ultimate expression of lysosomal enzymes because it is only after the construction of a mannose-6-phosphate recognition marker that the enzymes are recognized by a receptor and delivered to lysosomes. The five potential glycosylation sites (Asn-X-Ser/Thr) of the hexosaminidase β -chain were individually modified by site-directed mutagenesis and the constructs were expressed in Cos 1 cells under control of the SV-40 late promoter. By this analysis, we determined that four of the five potential glycosylation sites were modified by addition of oligosaccharide chains. The lack of any one of the oligosaccharides did not dramatically affect catalytic activity or the delivery of the enzyme to lysosomes. We also demonstrated a selectivity in the phosphorylation of the oligosaccharides on hexosaminidase B; two of the four oligosaccharide chains were predominantly phosphorylated. When these two phosphorylated sites were removed by mutagenesis, the enzyme was not delivered to lysosomes. This work has also clarified the peptide structure of the mature enzyme.

II. A hexosaminidase α chain cDNA has been cloned from a library prepared with the fibroblast mRNA of a patient with the adult form of Tay-Sachs disease. We are currently sequencing the clone to identify the mutation responsible for this form of the disease.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52014-01 GBB

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

CD4 Receptor Structure/Function Project

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

PI: R. Daniel Camerini-Otero Chief, GBB, NIDDK
Others: Richard L. Proia Senior Staff Fellow, GBB, NIDDK

COOPERATING UNITS (if any)

Cynthia Tiffet Medical Staff Fellow OD, CC

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Molecular Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.0

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

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

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

The T cell surface glycoprotein CD4 is not only a receptor for antigen recognition and immune system activation, but also the receptor for the human immunodeficiency virus (HIV). Studies have shown that the presence of CD4 is not only necessary but sufficient to render cells susceptible to HIV infection. Several recent reports have also demonstrated that soluble CD4 is able to selectively inhibit and neutralize HIV binding and infection of CD4+ cells.

Glycosylation of some glycoproteins has been shown to be required for their processing and transport to the cell surface. The primary sequence of CD4 indicates two potential glycosylation sites. A recent report utilizing tunicamycin to block glycosylation of T4+ cells suggests that glycosylation is necessary for cell surface expression of the receptor.

We have used site-directed mutagenesis to create a series of mutants incapable of glycosylation at one or both sites. These mutants were placed under the control of the SV 40 late promoter and transfected and expressed in COS 1 cells. By this analysis we were able to show that both glycosylation sites are utilized in the expression of CD4.

Additionally we have constructed a truncated mutant of CD4 lacking the transmembrane and cytoplasmic portions which will be used in overexpression of the protein for further structure-function analysis.

SUMMARY OF BRANCH ACTIVITIES

The Digestive Diseases Branch has two sections (Section on Gastroenterology and the Liver Diseases Section). The Liver Diseases Section has 2 senior physicians and 3 medical staff fellows; the Section on Gastroenterology has 3 senior physicians and 5 medical staff fellows. The Digestive Diseases Branch also has approximately 10 guest investigators.

Detailed summaries of the activities of each section precede the individual project reports. Both sections are engaged in investigations of basic biologic processes (e.g., hormone action, membrane transport, cellular and humoral immunology) and are attempting to apply this information to understand the pathophysiology of various disorders involving the liver and gastrointestinal tract. Both sections are also involved in attempts to improve therapy of clinical disorders such as neoplasms associated with overproduction of gastrointestinal hormones, hepatitis and fulminant hepatic failure.

Section on Gastroenterology

The Section on Gastroenterology is currently following approximately 120 patients with Zollinger-Ellison syndrome (ZES, gastrin-producing neoplasm, hypergastrinemia and increased secretion of gastric acid). All patients are currently being treated with oral medication that inhibits gastric acid secretion.

Although histamine H₂-receptor antagonists are effective inhibitors of gastric acid secretion in patients with Zollinger-Ellison syndrome, these agents must be taken in large doses and at frequent intervals. Omeprazole a new antisecretory agent that inhibits gastric H⁺,K⁺-ATPase was tested for therapeutic efficacy in patients with Zollinger-Ellison syndrome. A single dose of omeprazole inhibited gastric acid secretion for more than 48 hours in patients with Zollinger-Ellison syndrome. In 90% of patients with Zollinger-Ellison syndrome, gastric acid secretion could be adequately inhibited by a single daily dose of omeprazole. Omeprazole has been free of detectable toxicity during four years of therapy. Because of its long duration of action, omeprazole offers an advance in convenient medical therapy for Zollinger-Ellison syndrome compared with histamine H₂-receptor antagonists. To date we have treated 45 patients with omeprazole.

Previously we have shown that patients with Zollinger-Ellison syndrome can be adequately treated if gastric acid secretion is reduced to below 10 mEq/hr during the last hour before the next dose of medication. These results were obtained primarily in patients who had not undergone previous gastric surgery. During this past year we have found that in patients who have had previous gastric surgery gastric acid secretion must be reduced to below 5 mEq/hr during the last hour before the next dose of medication in order to prevent gastrointestinal pathology.

During the past 5 years we have evaluated chemotherapy with streptozotocin, 5-FU and adriamycin for patients with Zollinger-Ellison syndrome and metastatic gastrinoma. After treating 10 patients we have found that 40 percent of treated patients show an initial favorable response (no new tumor and > 25 percent decrease in size of lesions) that is not sustained. Sixty percent of treated patients do not respond to chemotherapy. Patients who show an initial response to chemotherapy do not live longer than those who do not respond. There is an obvious need for some new chemotherapeutic regimen for treating patients with metastatic gastrinoma.

During this past year we have found that pancreatic acinar cells possess gastrin receptors. These receptors have high affinities for gastrin as well as CCK in contrast to CCK receptors, which have a high affinity for CCK and a low affinity for gastrin. Future studies will be directed toward establishing the cell function that might be altered as a consequence of gastrin receptor occupation.

In all cell systems studied previously the receptors that interact with gastrin also interact with CCK and vice versa. In gastric chief cells gastrin and CCK each cause significant stimulation of pepsinogen secretion; however, the receptors that mediate the action of CCK do not interact with gastrin and the receptors that mediate the action of gastrin do not interact with CCK.

Previous studies have identified bombesin receptor antagonists with a low affinity for bombesin receptors on various cell types. Recently, however, we have shown that an analogue of bombesin with a reduced peptide bond between residues number 13 and 14 has a high affinity (Kd in mM range) for the bombesin receptor on pancreatic acinar cells.

Studies of the kinetics of binding of radiolabeled CCK-8 indicate that there are two states of binding of CCK to receptors in pancreatic acinar cells - a rapidly dissociating state and a slowly dissociating state. Binding in the rapidly dissociating state was maximal within 3 minutes, did not depend on incubation temperature or cellular energy metabolism, could be stripped by 0.5M potassium thiocyanate and showed accelerated dissociation by CCK receptor agonists and antagonists. Binding in the slowly dissociating state was maximal after 60 minutes, was decreased by reducing the incubation temperature or inhibiting cellular energy metabolism, was not stripped by 0.5M potassium thiocyanate and did not show accelerated dissociation by agents that occupy the CCK receptor.

Pancreatic acinar cells possess two classes of receptors that interact with CCK. One class has a high affinity for CCK and occupation of this class of receptors by CCK appears to cause activation of phospholipase C, increased turnover of inositol phospholipids, mobilization of cellular calcium and activation of protein kinase C. The other class has a low affinity for CCK and occupation of this class of receptors by CCK appears to cause activation of adenylate cyclase, increased cellular cyclic AMP and activation of protein kinase A. Pancreatic acinar cells thus appear to be unique in that in these cells the same hormone is able to activate both of the major intracellular signal pathways.

Liver Diseases Section

The Liver Diseases Section is currently responsible for eight principal studies.

I. Studies Relating to the Pathogenesis of Hepatic Encephalopathy

The abnormal pattern of visual evoked responses (VERs) in animals with hepatic encephalopathy (HE) due to fulminant hepatic failure (FHF) resembles that induced by drugs which promote GABA-ergic neurotransmission, including benzodiazepines (BZs). Furthermore, rabbits with HE due to FHF exhibit increased resistance to the convulsive effects of the GABA receptor antagonist, bicuculline. Ameliorations of HE (both clinical and electrophysiologic (VER waveform)) have been induced in animals with FHF by BZ receptor antagonists. Furthermore, spontaneous in vitro activity of Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. These findings suggest that in HE due to FHF: (i) There is increased GABA-ergic tone; (ii) Blockading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) An endogenous BZ receptor agonist may contribute to HE. Such a ligand is being isolated from supernatants of brain obtained from models of HE. [E.A. Jones, J. Vergalla, S.H. Gammal, P. Martin, N. Bergasa, B. Baker, M. Nam, M. Lisker-Melman, A. Basile, P. Skolnick, C. Banner].

II. Studies of Cellular Immune Function in Primary Biliary Cirrhosis

The role of abnormal immune mechanisms in the mediation of the hepatobiliary lesion of primary biliary cirrhosis (PBC) is being studied. Recently with the use of monoclonal antibodies it has become apparent that CD4 (T4) T cells can be subdivided into subpopulations having unique functions. In particular CD4 positive, Leu-8 positive T cells have been demonstrated to have direct suppressor function, as well as the capacity for inducing CD8 (T8) suppressor cells. In addition, it has been shown that the CD4 positive, Leu-8 positive T cell population is the predominant autoreactive T cell subpopulation in peripheral blood. Thus the activation of autoreactive cells and suppressor T cell function may involve common mechanisms mediated by a single T cell subset. Since a defect in suppressor function and a defect in the autologous mixed lymphocyte reaction have been shown to be present in patients with PBC, it seems likely that the function of the CD4 positive, Leu-8 positive T cell subset may be abnormal in patients with this disease. CD4+, Leu-8+ T cells from patients with PBC, but not from patients with other liver diseases, have been shown to exhibit a defect in their ability to suppress immunoglobulin synthesis by B cells in vitro. Furthermore the proliferative responses of these cells from patients with PBC to mitogenic stimulation was found to be impaired. However, the defect in proliferative responses did not correlate with the defect in suppression of immunoglobulin synthesis, suggesting that these two defects are due to different mechanisms. The abnormal function of the CD4+, Leu-8+ T cell subpopulation in patients with PBC may play a central role in the defective immunoregulation found in this disease. Exposure of this subpopulation of T cells from patients with PBC to phorbol ester, which induces protein kinase C, corrects the defective response

of these cells to mitogens. Thus abnormal function of the biochemical pathway involving protein kinase C may contribute to the immunological abnormalities exhibited by patients with PBC. [E.A. Jones, R. Moreno-Otero, T. Suou, M. Civeira, S.P. James, J.H. Hoofnagle, M.E. Kanof, J. Vergalla].

III. Studies of Protease Inhibitor (Pi) Phenotypes

Pi phenotypes and serum α -1-antitrypsin (α 1AT) concentrations have been determined in 80 unselected southern African Black patients with hepatocellular carcinoma and 103 age, sex and tribally matched control subjects. Non-MM phenotypes were present in 8.7% of patients with hepatocellular carcinoma and 12.6% of controls. The heterozygous PiZ carrier state was present in 5.0% of patients with hepatocellular carcinoma (i.e., 4) and 1.9% of controls; no subjects had the homozygous PiZZ phenotype. No patients with hepatocellular carcinoma had a subnormal serum α 1AT concentration as assessed by rocket immunoelectrophoresis. The four patients who had the heterozygous PiZ phenotype did not have fibrolamellar carcinomas. It is inferred that α 1AT deficiency does not play an etiologic role in hepatocellular carcinoma in southern African Blacks. [E.A. Jones, J. Vergalla, R. Crystal; not NIH: M.C. Kew].

IV. Controlled Trial of Chlorambucil Therapy in Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is a disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to non-suppurative, presumably autoimmune, destruction of small bile ducts. As immunosuppression with alkylating agents has been shown to be beneficial in certain autoimmune diseases, a controlled trial of chlorambucil therapy for patients with symptomatic PBC has been conducted. Twenty-four patients (23 women, 1 man; ages 34-63) were admitted to this trial: 13 were randomized to receive a four year course of chlorambucil therapy (0.5-4.0 mg/day) and 11 to receive no treatment. The dose of chlorambucil was adjusted to reduce the peripheral blood lymphocyte count by 50% and maintain the polymorphonuclear leukocyte count above 1000 per c.mm. During follow-up, two patients have died: both in the control group. The mean serum bilirubin levels remained almost constant in the treated group but increased by an average of about 50% each year in the control group. Mean serum albumin values increased slightly in treated patients but decreased in control patients. Mean serum aminotransferase levels became significantly less in treated patients than in controls. Mean serum immunoglobulin (IgM and IgG) levels decreased from elevated values to values within the normal range in all chlorambucil-treated patients, but did not change appreciably in control patients. Liver biopsy histopathology after one, and/or two years revealed significantly less inflammation, slightly less fibrosis and less progression of the stage of disease in the treated than in the control patients. Potential side effects of chlorambucil therapy included onset of menopause, localized herpes simplex or zoster and persistent leukopenia or thrombocytopenia. The results of this trial strongly suggest that chlorambucil therapy retards the progression of PBC. In an additional study vitamin E status and intestinal absorption are being studied in patients admitted to the chlorambucil trial. [E.A. Jones, J.H. Hoofnagle; not NIH: R.N.M. MacSween, R.J. Sokol, W.F. Balistreri.]

V. Studies of the Natural History and Treatment of Chronic Type B Hepatitis

A cohort of patients with chronic type B hepatitis is being evaluated and followed to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials in which antiviral or immunomodulatory agents are being administered. Eight patients were entered into a study of the treatment of chronic type B hepatitis with recombinant human alpha and gamma interferon. Alpha interferon has a more pronounced inhibitory effect than gamma interferon on serologic markers of HBV replication. Gamma interferon was associated with severe side effects which may limit its use. A randomized controlled trial of interferon therapy vs. no treatment is underway. In addition, a pilot study is examining the effect of one month of corticosteroid pre-treatment followed by interferon therapy for patients who have previously not responded to interferon therapy alone. [J.H. Hoofnagle, A.M. Di Bisceglie, C. Kassianides, P. Martin, N. Bergasa, J. Korenman, M. Lisker-Melman, E.A. Jones; not NIH: J. Gerin, S. Order, M. Sjogren].

VI. Studies of the Natural History and Treatment of Chronic Non-A, Non-B Hepatitis

Patients with well-documented chronic non-A, non-B hepatitis are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients is available to evaluate experimental therapies for this disease. A pilot study demonstrated that alpha interferon was effective in normalizing serum ALT activities in a majority of cases. This effect was associated with an improvement in liver histopathology (decreased activity of hepatitis). A prospective randomized, placebo-controlled trial of alpha interferon therapy for chronic non A, non B hepatitis is underway. Forty one patients have been entered and 26 have so far completed 6 months of therapy. [J.H. Hoofnagle, A.M. Di Bisceglie, C. Kassianides, M. Lisker-Melman, N. Bergasa, J. Korenman, H.J. Alter, J. Everhart, E.A. Jones; not NIH: Z. Goodman].

VII. Immunologic Studies of Chronic Viral Hepatitis

Immunological factors seem to be important in determining the course and outcome of both acute and chronic viral hepatitis. Furthermore promising therapies for chronic viral hepatitis have profound effects on immune function and sustained responses to therapy may depend largely on restoration of normal immune responsiveness. The role of immunologic mechanisms in determining the course and ultimate outcome of viral hepatitis is being studied and the effects of antiviral and immunomodulatory therapies on the immune system are being evaluated. Serial studies of cellular immune function have been performed on patients with chronic type B hepatitis treated with interferon. [J.H. Hoofnagle, E.A. Jones, A.M. Di Bisceglie, C. Kassianides, M. Lisker-Melman, R. Moreno-Otero, J. Korenman, J. Ambrus; not NIH: T. Cupps].

VIII. Studies of the Natural History and Treatment of Duck Hepatitis B Virus Infection

Duck hepatitis B virus (DHBV) infection is a potentially useful experimental model of human hepatitis B virus infection. New antiviral and immunomodulatory agents are being assessed for their ability to suppress DHBV replication in ducks. It is anticipated that the ability of a drug to suppress DHBV replication will be shown to be a satisfactory screening test for new effective therapies for chronic type B hepatitis in man. Care of DHBV-infected ducks as well as methods for obtaining serum and liver tissue from ducks have been standardized. Reproducible assays for quantitating DHBV DNA and DNA polymerase in serum have been established. 2',3'-dideoxycytidine has been shown to be a potent inhibitor of DHBV replication; its anti-viral effect is comparable to that of adenine arabinoside monophosphate. The effect of 2', 3'-dideoxyadenine on DHBV replication is currently being evaluated. [E.A. Jones, C. Kassianides, P. Martin, H. Robcis, R. Miller, H. Mitsuya, S. Broder, J.H. Hoofnagle].

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53001-18 DDB

PERIOD COVERED

October 1, 1987 to Sept. 30, 1988

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

Studies of Membrane Function

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

PI:	Jerry D. Gardner	Chief	DDB, NIDDK
Others:	R. T. Jensen	Senior Investigator	DDB, NIDDK
	P. N. Maton	Visiting Scientist	DDB, NIDDK
	S. A. Wank, R. Vinavek, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	L. Miller, H. Stark, Z. Saeed	Medical Staff Fellows	DDB, NIDDK
	H-C. V. Chiang, J. London	Medical Staff Fellows	DDB, NIDDK
	Z-C. Zhou, D-H. Yu, L. Zhang	Visiting Fellows	DDB, NIDDK
	D. Menozzi, T. von Schrenck	Visiting Fellows	DDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5.0

PROFESSIONAL:

3.6

OTHER:

1.6

CHECK APPROPRIATE BOX(ES)

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

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

The broad categories which are included in the project are: 1) Characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53002-16 DDB

PERIOD COVERED

October 1, 1987 to Sept. 30, 1988

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

Gastrointestinal Hormones

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

PI:	J. D. Gardner	Chief	DDB, NIDDK
Others:	R. T. Jensen	Senior Investigator	DDB, NIDDK
	P. N. Maton	Visiting Scientist	DDB, NIDDK
	S. Wank, R. Vinayek, H. Frucht, L. S. Zaeed, V. Chiang, H. Stark	Miller, J. London, Medical Staff Fellows	DDB, NIDDK
	Z-C. Zhou, D-H. Yu, L. Zhang	Visiting Fellows	DDB, NIDDK
	M. Younes, D. Menozzi, S-C. Huang	Visiting Fellows	DDB, NIDDK
	S. Sato	Visiting Associate	DDB, NIDDK

COOPERATING UNITS (if any)

Dept. of Chemistry, Case-Western Reserve Univ., Cleveland, Ohio
Div. of Cellular Biology, Kennedy Institute for Rheumatology, London, England

LAB/BRANCH

Digestive Diseases Branch

SECTION

Section on Gastroenterology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

5.6

PROFESSIONAL:

4.0

OTHER:

1.6

CHECK APPROPRIATE BOX(ES)

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

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

In vitro systems are being used to study the mechanism of action of gastrin, secretin, cholecystokinin, bombesin, substance P and vasoactive intestinal peptide with their specific membrane receptors.

Clinical investigators are directed toward developing alternative forms of therapy for and elucidating the pathogenesis of disorders characterized by ectopic production of gastrointestinal hormones (e.g., Zollinger-Ellison syndrome and pancreatic cholera).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53004-16 DDB

PERIOD COVERED

October 1, 1987 to Sept. 30, 1988

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

Cyclic Nucleotide Mediated Functions

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

PI:	Jerry D. Gardner	Chief	DDB, NIDDK
Others:	R. T. Jensen	Senior Investigator	DDB, NIDDK
	P. N. Maton	Visiting Scientist	DDB, NIDDK
	S. Wank, R. Vinavek, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	Z-C. Zhou, D-H. Yu, L. Zhang	Visiting Fellows	DDB, NIDDK
	M. Younes, D. Menozzi,	Visiting Fellows	DDB, NIDDK
	T. von Schrenck, S-C. Huang	Visiting Fellows	DDB, NIDDK
	S. Mantey, C. Sharp	Chemists	DDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Gastroenterology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

6.6

PROFESSIONAL:

5.0

OTHER:

1.6

CHECK APPROPRIATE BOX(ES)

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

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

In vitro systems are being used to characterize the mechanism by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53501-15 DDB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Studies Relating to the Pathogenesis of Hepatic Encephalopathy

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

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J. Vergalla	Chemist	LDS, NIDDK
	S.H. Gammal	Guest Researcher	LDS, NIDDK
	P. Martin	Visiting Associate	LDS, NIDDK
	M. Ferreira	Guest Researcher	LDS, NIDDK
	M. Nam	NRSA Fellow	LDS, NIDDK
	M. Lisker-Melman	Visiting Associate	LDS, NIDDK
	N. Bergasa	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (if any)

Laboratory of Neuroscience, NIDDK (P. Skolnick and A. Basile)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

The abnormal pattern of visual evoked responses (VERs) in animals with hepatic encephalopathy (HE) due to fulminant hepatic failure (FHF) resembles that associated with encephalopathy induced by drugs which promote GABA-ergic neurotransmission, including benzodiazepines (BZs). These findings suggest that the pattern of neuronal activity in HE may resemble that associated with activation of the GABA inhibitory neurotransmitter system. Furthermore, rabbits with HE due to FHF exhibit increased resistance to the convulsive effects of the GABA receptor antagonist, bicuculline. Ameliorations of HE (both clinical and electrophysiologic (VER waveform)) have been induced in animals with FHF by BZ receptor antagonists. Furthermore, spontaneous in vitro activity of Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. These findings suggest that in HE due to FHF: (i) There is increased GABA-ergic tone; (ii) Blockading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) An endogenous BZ receptor agonist may contribute to HE. Such a ligand is being isolated from supernatants of brain obtained from models of HE.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 53503-14 DDB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Immunologic Studies of Primary Biliary Cirrhosis

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

PI: E.A. Jones Chief LDS, NIDDK

Others: J.H. Hoofnagle Clinical Director NIDDK
 J. Vergalla Chemist LDS, NIDDK
 T. Suou Guest Researcher LDS, NIDDK
 R. Moreno-Otero Guest Researcher LDS, NIDDK

COOPERATING UNITS (if any)

Laboratory of Tumor Cell Biology, NCI (M. Civeira)
 Laboratory of Clinical Investigation, NIAID (S.P. James and M.E. Kanof)

LAB/BRANCH Digestive Diseases Branch

SECTION Liver Diseases Section

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 2.0	OTHER:
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CHECK APPROPRIATE BOX(ES)

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

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

Primary biliary cirrhosis (PBC) appears to be a model autoimmune disease. Abnormal immune mechanisms are being studied in this disease, but so far a disease-specific immunologic deficit has not been defined with certainty. Recently recognized defects of humoral immunity include: (i) Evidence for the existence of an expanded clone of activated B cells that synthesize mitochondrial antibodies with different antigenic specificities from those synthesized by normal B cells; and (ii) Detection of a serum factor, probably an abnormally immunoreactive IgM, which blocks the binding of C3b-opsonized erythrocytes by monocytes. The latter finding affords a potential explanation for the C3b-receptor specific clearance defect by fixed macrophages in PBC. Recently recognized defects in cellular immunity include: (i) A diminished ability of patient T cells to suppress immunoglobulin synthesis; (ii) The presence of increased numbers of circulating activated B cells; and (iii) Hyporeactivity of lymphocytes in the autologous mixed lymphocyte reaction, which normally leads to activation of suppressor T cells. To determine whether such abnormalities of lymphocyte function in PBC might be due to altered function of immunoregulatory T cell subpopulations, phenotypic and functional characteristics of T cells that have the CD4 antigen detectable (by monoclonal antibody) on their surface were examined. In contrast to normal subjects and patients with other liver diseases, patients with PBC were found to have defects in CD4+, Leu-8+ T cell-mediated suppression of immunoglobulin synthesis and mitogen-stimulated proliferation. These defects may play a central role in the defective immunoregulation found in this disease.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53505-13 DDB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Studies of Alpha-1-Antitrypsin Phenotypes and Metabolism

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

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J. Vergalla	Chemist	LDS, NIDDK
	R. Crystal	Medical Officer	PB, NHLBI

COOPERATING UNITS (if any)

University of the Witwatersrand, Johannesburg, South Africa
(Dr. M. C. Kew)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.25

PROFESSIONAL

0

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

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

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

Protease inhibitor (Pi) phenotypes have been determined using isoelectric focusing on polyacrylamide gel in populations of normal subjects and patients with rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome and hepatocellular carcinoma. Of 80 unselected southern African Black patients with hepatocellular carcinoma, the incidence of aberrant (non-MM) phenotypes was 8.7%. In 103 age, sex and tribally-matched control subjects the corresponding incidence was 12.6%. None of the patients or controls had the PiZZ phenotype. 5% of patients and 1.9% of controls were heterozygous carriers of the Z gene. No patient with hepatocellular carcinoma had a sub-normal serum concentration of alpha-1-antitrypsin. The four patients with the heterozygous Z phenotype did not have fibrolamellar carcinomas. These findings suggest that alpha-1-antitrypsin deficiency does not play an etiologic role in hepatocellular carcinoma in southern African Blacks.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53508-11 DDB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Studies of Hepatic Receptors for Glycoproteins

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

PI: E.A. Jones Chief LDS, NIDDK

Others: J. Vergalla Chemist LDS, NIDDK

COOPERATING UNITS (if any)

Laboratory of Biochemistry and Metabolism, NIDDK (G. Ashwell)

LAB/BRANCH Digestive Diseases Branch

SECTION Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.25

PROFESSIONAL:

0.25

OTHER:

0

CHECK APPROPRIATE BOX(ES)

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

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

The cellular location and carbohydrate specificities of a glycoprotein recognition system on rat hepatic sinusoidal cells have been determined. Purified preparations of endothelial, Kupffer and parenchymal cells have been prepared by in situ collagenase liver perfusion, centrifugation on Percoll gradients and centrifugal elutriation. ^{125}I -labeled agalactoorosomucoid (AGOR), an N-acetylglucosamine-terminated glycoprotein, was selectively and specifically taken up in vitro by endothelial cells. Glucose and a glucose-albumin conjugate competitively inhibited this uptake process over a wide range of concentrations. Uptake by cells from fasted rats was enhanced, but uptake by cells from fasted or fed diabetic rats was normal. The in vivo hepatic uptake and catabolism of ^{125}I -AGOR were slower in diabetic than normal rats. It is inferred that 1) the hepatic receptors which recognize N-acetylglucosamine/mannose terminated glycoproteins are located predominantly on endothelial cells, 2) these receptors are glucose sensitive, 3) fasting increases the number of these receptors and 4) diabetes mellitus abolishes this effect of fasting and impairs the function of this receptor in vivo. These findings suggest a mechanism for abnormal glycoprotein metabolism in diabetes mellitus. This carbohydrate recognition system may play an important role in the removal of potentially autodestructive glycoprotein lysosomal hydrolases and other glycoprotein enzymes from the circulation under normal physiological conditions and in disease states.

NOTICE OF INTRAMURAL RESEARCH PROJECT

301 DK 53509-10 DDB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Studies of the Natural History and Treatment of Chronic Type B Hepatitis

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

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Clinical Director	NIDDK
	C. Kassianides	Visiting Associate	LDS, NIDDK
	M. Lisker-Melman	Visiting Associate	LDS, NIDDK
	A. Di Bisceglie	Visiting Associate	LDS, NIDDK
	P. Martin	Visiting Associate	LDS, NIDDK
	N. Bergasa	Medical Staff Fellow	LDS, NIDDK
	J. Korenman	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (if any)

Georgetown University, Washington, D.C. (J. Gerin)
 Johns Hopkins University, Baltimore (S Order)
 Walter Reed Army Institute of Research, Washington, D.C. (M. Sjogren)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

3

PROFESSIONAL:

2

OTHER:

1

CHECK APPROPRIATE BOX(ES)

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

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

A cohort of patients with chronic type B hepatitis is being evaluated and followed to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials of antiviral and/or immunomodulatory agents. Efforts are now being directed towards improving the response rate to interferon alone. A pilot study of alpha and gamma interferon in combination has been completed. While alpha interferon induced a dose-dependent inhibition of viral DNA polymerase activity, gamma interferon had little or no effect on this enzyme. In addition, gamma interferon was associated with more severe side effects than alpha interferon. When used in combination, no additive or synergistic antiviral effects were apparent. Two new studies using alpha interferon as therapy for chronic type B hepatitis are underway. The first is a randomized, controlled trial to reevaluate the effects of alpha interferon alone (at a dose of 10 million units three times weekly) compared to no therapy in patients without factors that may potentially interfere with the therapeutic response. So far five patients have been entered and no results are yet available. A second study is designed for patients who have not responded to interferon alone in previous studies. In these cases, the effect of pretreatment with a 4 week course of prednisone before administration of interferon is being evaluated. It is hoped that the immunostimulatory effects of rapid withdrawal of corticosteroids, by inducing an exacerbation of hepatitis activity will tend to optimize the antiviral effects of alpha interferon. Six patients have been entered into this study so far, none has yet completed the treatment.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53510-09 DDB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Studies of the Natural History and Treatment of Chronic Non-A, Non-B Hepatitis

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

PI: E.A. Jones Chief LDS, NIDDK

Others:	J.H. Hoofnagle	Clinical Director	NIDDK
	A. Di Bisceglie	Visiting Associate	LDS, NIDDK
	C. Kassianides	Visiting Associate	LDS, NIDDK
	M. Lisker-Melman	Visiting Associate	LDS, NIDDK
	J. Korenman	Medical Staff Fellow	LDS, NIDDK
	N. Bergasa	Medical Staff Fellow	LDS, NIDDK

COOPERATING UNITS (if any)

NIH Blood Bank (H.J. Alter)
 Division of Digestive Diseases and Nutrition, NIDDK (J. Everhart)
 Armed Forces Institute of Pathology, Washington, D.C. (Z. Goodman)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

3

PROFESSIONAL

2

OTHER

1

CHECK APPROPRIATE BOX(ES)

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

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

Patients with well-documented chronic non-A, non-B (NANB) hepatitis are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients are available to evaluate experimental therapies for this disease. In a pilot study, 10 patients were treated with alpha-interferon. Follow up shows that 6 seem to have had a long-term apparently complete remission of the hepatitis. In 2 cases the response was favorable but temporary and 2 patients did not respond at all. A prospective, randomized, placebo-controlled, double-blind trial of a six month course of human alpha interferon in patients with chronic NANB hepatitis is underway. Forty one patients have been entered and 26 have completed 6 months of treatment so far. 12 of 13 patients treated with interferon showed some decrease in serum ALT activity and in 7 of these, ALT returned to normal levels after treatment. Mean serum ALT activity did not become normal in any placebo-treated patients. Interferon was generally well tolerated and similar untoward developments were noted in interferon and placebo treated groups.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53511-09 DDB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Controlled Trial of Chlorambucil in Primary Biliary Cirrhosis

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

PI: E.A. Jones Chief LDS, NIDDK

Others: J.H. Hoofnagle Clinical Director NIDDK

COOPERATING UNITS (if any)

Pediatric Services, Universities of Denver and Cincinnati (R.J. Sokol;
W.F. Balistreri).

LAB/BRANCH Digestive Diseases Branch

SECTION Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

.25

PROFESSIONAL:

.25

OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Primary biliary cirrhosis (PBC) is a disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to non-suppurative, presumably autoimmune, destruction of septal and the larger interlobular bile ducts. Because certain other autoimmune diseases appear to respond favorably to alkylating agents, a controlled trial of chlorambucil therapy for patients with symptomatic PBC has been conducted. Twenty-four patients were admitted to this trial: 13 were randomized to receive chlorambucil therapy (0.5-4.0 mg/day) and 11 to receive no treatment. The dose of chlorambucil was adjusted to reduce the peripheral blood lymphocyte count by 50% and maintain the polymorphonuclear leukocyte count above 1000 per c.mm. During follow-up, two patients died: both were controls. Mean serum bilirubin levels decreased slightly in the treated group but increased significantly in the controls. By 2 years the mean serum albumin had increased significantly in treated patients but decreased in controls. Mean serum transaminase levels became significantly less in treated patients than in controls. Mean serum immunoglobulin (IgM and IgG) levels decreased from elevated values to values within the normal range in all chlorambucil-treated patients, but remained elevated in controls. Liver biopsy histopathology after one and/or two years revealed significantly less inflammation, slightly less fibrosis and less progression of the stage of disease in the treated than in the control patients. Potential side effects of chlorambucil therapy included the onset of menopause in two patients, localized herpes simplex or zoster in 3 and, in 4 patients, persistent leukopenia or thrombocytopenia requiring discontinuation of the drug. These findings strongly suggest that chlorambucil therapy retards the progression of PBC, and that a search for safer (e.g. noncarcinogenic) and more effective immunosuppressive regimes for the treatment of this disease is likely to be rewarding.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

201 DK 53514-05 DDB

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

Immunological Studies in Chronic Viral Hepatitis

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

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	Jay H. Hoofnagle	Clinical Director	NIDDK
	M. Lisker-Melman	Visiting Associate	LDS, NIDDK
	A. Di Bisceglie	Visiting Associate	LDS, NIDDK
	T. Suou	Guest Researcher	LDS, NIDDK
	R. Moreno-Otero	Guest Researcher	LDS, NIDDK

COOPERATING UNITS (if any)

Laboratory of Immunoregulation, NIAID (Dr. Julian Ambrus)
Georgetown University (Dr. Tom Cupps)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

1.5

OTHER:

.5

CHECK APPROPRIATE BOX(ES)

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

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

Immunological factors seem to be important in determining the course and outcome of both acute and chronic viral hepatitis. Furthermore, promising therapies for chronic viral hepatitis have profound effects on immune function and sustained responses to therapy may depend largely on restoration of normal immune responsiveness. The role of immunologic mechanisms in determining the course and ultimate outcome of viral hepatitis is being studied and the effects of therapies on the immune system are being evaluated. Serial studies of cellular immune function have been conducted on patients with chronic type B hepatitis. In addition, the immunological status of patients with chronic type B hepatitis is being assessed in detail and the effects of immunosuppressive as well as antiviral therapy on immunological function in these patients are being studied prospectively.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 53515-02 DDB

PERIOD COVERED

October 1, 1987 through September 30, 1988

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

Studies of the Natural History and Treatment of Duck Hepatitis B Virus Infection

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

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	C.K. Kassianides	Visiting Associate	LDS, NIDDK
	J.H. Hoofnagle	Clinical Director	NIDDK
	P. Martin	Visiting Associate	LDS, NIDDK

COOPERATING UNITS (if any)

Comparative Animal Unit (H. Robcis)
 Hepatitis Virus Section, NIAID (R. Miller)
 Clinical Oncology Program, NCI (H. Mitsuya, S. Broder)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2

PROFESSIONAL:

1.75

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

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

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

There are many similarities in structure and properties between the human hepatitis B virus (HBV) and the duck hepatitis B virus (DHBV). These similarities suggest that DHBV infection in ducks may be a useful experimental model of human HBV infection, particularly as HBV cannot be grown readily in cell culture. Ducks infected with DHBV at birth become chronic carriers of the virus, although they may not develop overt hepatitis. Some DHBV-infected ducks have been reported to develop hepatocellular carcinoma, a tumor strongly linked etiologically in humans with chronic hepatitis B infection. As a screening test for new effective therapies for chronic type B hepatitis in man, new antiviral immunomodulatory agents are being assessed for their ability to suppress DHBV replication in ducks.

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ANNUAL REPORT OF THE

MOLECULAR, CELLULAR, AND NUTRITIONAL ENDOCRINOLOGY BRANCH

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The MCNEB continues basic and clinical investigations in the areas of molecular regulation and neuroendocrinology (Molecular Regulation and Neuroendocrinology Section, Bruce D. Weintraub, Chief); experimental diabetes, metabolism and nutrition (Experimental Diabetes, Metabolism and Nutrition Section, Samuel W. Cushman, Chief); and growth and development (Growth and Development Section, Matthew M. Rechler, Chief). The Branch has had many visiting fellows and associates, as well as international collaborations with the University of Milan, Italy; University of Marseilles, France; Karolinska Institute, Sweden; Institute of Organic Chemistry, Padova, Italy; Nankai University, Tianjin, Peoples Republic of China; Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, CSSR; Postgraduate School of Obstetrics and Gynaecology, University of Auckland, New Zealand; University of Naples, Italy; Department of Medicine, University of Gothenburg, Sweden; Endocrine Institute, Rambam Medical Center, Haifa, Israel; Department of Biochemistry, The University of Newcastle upon Tyne, England.

I. GLYCOPROTEIN HORMONES: MOLECULAR BIOLOGY, SYNTHESIS, PROCESSING, REGULATION, ACTION AND PATHOPHYSIOLOGY

A. Effect of Secretagogues on the Glycosylation of TSH

In order to develop a pituitary culture model that was dynamically responsive to secretagogues, we compared the secretory responsiveness of intact rat hemipituitaries to the response obtained in mouse thyrotropic tumor cells dispersed by the method of Vale *et al.* (Endocrinology 91:562, 1972) which uses multiple enzymes, and by the method of Hopkins and Farquhar (J. Cell Biol. 59:276, 1973), which uses mild trypsin treatment and mechanical disruption. In addition, we studied the secretory responsiveness of normal and hypothyroid rat pituitaries dispersed by the trypsin method. Intact rat hemipituitaries and dispersed mouse thyrotropic tumor cells showed a similar TSH secretory response to TRH (10^{-7}) of approximately 1.5 to 2-fold greater than control by RIA over a 24 h period. In contrast, normal rat pituitary cells dispersed by the trypsin method showed a 7-fold TSH response to TRH above control by RIA over 48 h. Responsiveness was noted as early as 1 h after administration of TRH, suggesting that TRH in these cells affected acute release and then mediated a prolonged stimulatory effect. There was no net increment in TSH biosynthesis by RIA over the course of these studies. Similarly, other agents known to affect TSH₂ secretion were studied. Phorbol 12-myristate 13-acetate (TPA; 5×10^{-7} M), which is able to activate protein kinase C directly, had a similar effect on TSH secretion measured by RIA, as did 5 mM 8-bromo cAMP, and depolarizing concentrations of KCl (60 mM). The kinetic pattern of release, net TSH responsiveness, and absent biosynthetic response was similar for the concentration of agents employed (above).

The effect of these agents on TSH glycosylation was then studied. It was discovered that rat pituitary cells dispersed by the method of Hopkins and Farquhar showed no potential for de novo biosynthesis of TSH. Nonetheless, they maintained a brisk secretory response to all of the agents previously mentioned. To determine whether secretagogues altered TSH carbohydrate structure, [³H]glucosamine-labeled TSH was immunoprecipitated and α- and β subunits were separated, enzymatically deglycosylated with endo F, and purified oligosaccharides were chromatographed by anion-exchange HPLC and concanavalin A-agarose affinity chromatography. Labeled oligosaccharides from secreted TSH released under control conditions, 10⁻⁷ M TRH, and 60 mM KCl had similar profiles, with 6 major species on TSH-α and 8-9 species with greater negative charge on TSH-β. In the presence of 5 mM 8-bromo-cAMP, however, there were noted forms that showed approximately a 2-fold increase in sialylation relative to sulfation. These studies suggest that cyclic nucleotides may be able to stimulate terminal sialylation and/or promote the secretion of TSH molecules enriched in sialic acid. In subsequent experiments we have shown that rat pituitary cells studied on day 2 after dispersion₃ display ongoing TSH biosynthesis. By continuous labeling with [³H]glucosamine, therefore, we are now studying the potential effect of these secretagogues on TSH carbohydrate biosynthesis, sorting and secretion.

. . . . N. Gesundheit, B. D. Weintraub

B. Developmental Regulation of TSH Carbohydrate Structure

Research performed during the past year has studied alterations in the carbohydrate structure of secreted rat thyrotropin (TSH) subunits in the perinatal hypothyroid state. Using Concanavalin A (Con A) Sepharose chromatography we have demonstrated that fewer oligosaccharides on TSHβ bound to Con A than TSHα for both perinatal hypothyroid and control animals, demonstrating more multiantennary chains on TSHβ. Anion exchange HPLC separation showed that TSH-β contained more negatively charged species compared to TSHα in both groups. In addition, hypothyroid rats showed a striking increase in relative sialylation of TSH oligosaccharides compared to control rats. The ratio of sialylated (N) to sulfated (S) species N1/S1, N2/S2 for TSHα were 2.3 and 5.1-fold greater, respectively, in hypothyroid animals. These ratios for TSHβ were 5.8 and 7.8 greater, respectively.

These data demonstrate that TSHβ from perinatal rats contains more highly branched and negatively charged oligosaccharides than TSHα. In addition, hypothyroid perinatal rats contain oligosaccharides enriched in sialic acid residues on both TSHα and TSHβ compared to control rats. These findings suggest that thyroid hormone regulates, at least in part, the sialylation of TSH, which may influence the metabolic clearance and bioactivity of this hormone. Further studies are now in progress to investigate the long-term effects of congenital hypothyroidism in adult animals.

. . . . P. W. Gyves, N. Gesundheit, B. D. Weintraub

C. Endocrine Regulation of TSH Carbohydrate Structure

We have previously shown that the carbohydrate moiety of secreted mTSH contains both sialic acid and sulfate residues. We have also previously shown that TSH from hypothyroid rats is cleared more slowly in euthyroid animals. To determine whether changes in carbohydrate structure might explain these differences in clearance, we studied TSH from normal mice and from mice at two stages of hypothyroidism. Pituitaries from normal adult male LAF mice and those from early (3 months after radiothyroidectomy) and late (12 months) hypothyroidism were incubated with [³H]glucosamine for 24 h. Labeled secreted TSH was immunoprecipitated and the α and β subunits were separated on SDS-PAGE and treated with endoglycosidase F to release the carbohydrate chains. Anion-exchange HPLC chromatograms showed seven distinct species in the α subunit carbohydrate, corresponding to the number of sialic acid (N) or sulfate (S) residues. Neuraminidase treatment abolished those peaks attributed to sialic acid. The singly sialylated (N1) to singly sulfated (S1) ratios (N1:S1) were 1.54, 1.47 and 3.53 for normal, early and late hypothyroidism, respectively. The N2:S2 ratios were 2.32, 1.99 and 9.53 for the same groups. Additionally, hypothyroidism of 12 months duration caused the appearance of a new tri-sialylated species (N3). This N3 form was barely detectable in the early hypothyroid mTSH and absent in the mTSH of normal animals. Comparing mTSH obtained from animals with hypothyroidism of 12 months duration to that of normal animals, our analysis showed two trends in the α subunit: increasing sialylation and decreasing sulfation. We conclude that increases in sialylation and decreases in sulfation of mTSH carbohydrate occur during prolonged hypothyroidism. These changes in TSH sialylation and sulfation may explain the previously observed decreases in the metabolic clearance rate of TSH derived from pituitaries of hypothyroid rats.

. . . . G. S. DeCherney, P. W. Gyves, N. Gesundheit, B D. Weintraub

D. Effects of Inhibitors of Carbohydrate Processing on TSH Secretion

We have previously shown that treatment of hypothyroid mouse pituitaries in primary culture with l-deoxynojirimycin, an inhibitor of glucosidases I and II, delayed the attainment of endo H resistance (signifying final complex carbohydrate structure) for both TSH and free α while having no effect on subunit combination. l-deoxynojirimycin reduced the amount of secreted TSH to 17% of control while reducing free α secreted to only 65% of control. Both basal differences in relative rates of TSH and free α processing and secretion as well as l-deoxynojirimycin induced differences suggest separate secretory pathways which may be determined by the carbohydrate structure.

In current studies performed with dispersed rat pituitary cells, we have begun to explore the effects of other carbohydrate inhibitors on the processing and secretion of TSH. Castanospermine, an inhibitor of glucosidase I, bromoconduritol and N-methyl-l-deoxynojirimycin, inhibitors of glucosidases I and II, reduced secretion of TSH, comparable to that produced by l-deoxynojirimycin. In contrast, l-deoxymannojirimycin, an inhibitor of mannosidase I, and swainsonine, an inhibitor

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of mannosidase II, both increased the rate of TSH secretion. These studies suggest that later carbohydrate processing affects TSH sorting and secretion in a different manner from early processing.

. . . . B. S. Stannard, N. Gesundheit, B. D. Weintraub

E. Molecular Cloning of a cDNA Encoding a Human Thyrotropin (hTSH) receptor

The actions of hTSH as well as thyroid-stimulating immunoglobulins (TSI) are thought to be mediated through a common plasma membrane receptor whose structure has not yet been determined. To elucidate the structure of the hTSH receptor, a human thyroid carcinoma λ gt11 cDNA library was screened with monoclonal antibodies derived from patients with Graves' disease. A clone was identified, whose expressed fusion protein bound to polyclonal TSI but not to control sera. This protein also bound to ¹²⁵I-bovine TSH and was displaced with high affinity by both unlabeled bovine and hTSH. In contrast, insulin and prolactin at 10^{-6} M showed minimal displacement, while hCG, hLH and hFSH showed low affinity displacement. Sequencing of this cDNA revealed an open reading frame coding for a protein with seven hydrophobic domains analogous to other guanine-nucleotide coupled receptors. Northern analysis identified a 2.8 kb mRNA species in human thyroid tissue, but not in human liver or brain. In summary: 1) A cDNA clone was isolated whose expressed fusion protein bound to monoclonal and polyclonal TSI and to TSH with high affinity and with specificity expected for the hTSH receptor. 2) DNA sequencing revealed a protein with the structure of an integral membrane protein. 3) mRNA expression of this putative hTSH receptor displayed tissue specificity. These data suggest that a cDNA encoding a receptor which interacts with both hTSH and TSI has been isolated.

. . . . T. Yoshida, F. E. Wondisford, B. D. Weintraub

F. Molecular Cloning of the Gene Encoding a Human TSH Receptor

We have recently isolated a cDNA from a human well-differentiated thyroid expression library which codes for a protein with properties of the TSH receptor (see above). In the current study we have cloned this TSH receptor gene to further our understanding of its structure and expression. An EMBL 3 human leukocyte genomic library was screened with a 600 bp cDNA coding segment of the TSH receptor used as a probe, and one positive clone from 5×10^6 plaques was obtained.

A 7.0 kbp Bam HI segment of this insert that alone hybridized to the 600 bp cDNA probe was subcloned. This subclone on Eco RI digestion revealed an approximately 600 bp segment that hybridized to the 600 bp cDNA probe. This 600 bp genomic RI segment was flanked by 2.3 kbp and 4.1 kbp genomic segments. The recombinant phage (and genomic subclones) did not hybridize to a 400 bp cDNA segment. Because the 600 bp and 400 bp cDNAs are contiguous, these data suggested there must be at least one intervening sequence between these exonic regions.

Sequencing of the 600 bp genomic RI fragment is now in progress, and the result will be compared with the sequence of two TSH receptor cDNAs presently being analyzed. Further genomic Southern analysis and genomic

cloning using a putative full-length TSH receptor cDNA as probe are in progress. Recent experiments have raised the interesting possibility of a family of TSH receptor genes.

. . . . S. J. Usala, P. E. Wondisford, T. Yoshida, B. D. Weintraub

G. Enzymatic Deglycosylation of Thyrotropin

Chemical deglycosylation of thyrotropin (TSH) and gonadotropins virtually abolishes their bioactivity but not their receptor-binding activity. Recent reports from this as well as other laboratories have suggested that enzymatic deglycosylation with peptide-N-glycosidase F or endoglycosidase F (endo F) resulted in less dramatic changes in hormonal activity. However, in the above studies, complete removal of all glycan chains was not achieved. In the present study we attempted to achieve complete deglycosylation of TSH and its subunits using endo F under nondenaturing conditions. We characterized the deglycosylated (dg) products by SDS-PAGE under reducing conditions. In the bioassay, the dg rat TSH showed a 60% decrease in activity compared to control. However, on concanavalin A affinity chromatography, about 70% of the dg rat TSH bound to the lectin, thus showing that the carbohydrate removal was incomplete and the estimation of deglycosylation from SDS-PAGE was misleading. Subunits of bovine TSH were more readily dg by endo F and much more so in the presence of Nonidet P-40 as assessed by SDS-PAGE analysis. Concanavalin A chromatograph of the dg β subunit confirmed that all dg material applied did not bind to the lectin showing complete removal of the glycan chains. These results suggest that prior studies may have overestimated the amount of deglycosylation achieved by endo F treatment of intact hormones. Deglycosylation of subunits followed by recombination appears necessary to obtain complete deglycosylation and to assess the role of carbohydrate in hormone action. Using such methods we have found that enzymatically deglycosylated TSH was less than 5% as active as native TSH, levels comparable to chemically deglycosylated hormone.

. . . . R. Thotakura, P. W. Gyves, B. D. Weintraub

H. Isolation and Characterization of the Human Thyrotropin Beta Subunit Gene

The human thyrotropin β -subunit gene was isolated and characterized from two genomic libraries and found to contain three exons separated by two introns of 3.9 and .45 kilobase pairs. Exon two and three in the mouse thyrotropin β -subunit gene are not found in humans due to a lack of consensus sequences important in exon splicing. Moreover, using primer extension, RNA sequencing, and S1 nuclease analysis we determined, in a TSH-producing pituitary adenoma, that exon 1 in humans contains only one transcriptional start site and is 37 base pairs in length. This is unlike both the rat and mouse thyrotropin β -subunit gene which contain two transcriptional start sites. Changes in the genomic structure of the more 5' "TATA box" and surrounding "CAAT box" might explain why the more 5' start site in humans is apparently not utilized. Moreover, the first exon in human is longer than the corresponding exon in murine species presumably due to a 9 base pair insertion between the "TATA box"

and transcriptional start site (37 vs 27 nucleotides). Thus while alternative exon splicing and differential start site utilization in response to thyroid hormone may be important in the regulation of murine thyrotropin β -subunit genes, they are not found in man.

. . . . F. E. Wondisford, J. M. Moates, B. D. Weintraub

I. Thyroid Hormone Regulation of the TSH β Gene

A comparison of 5' flanking DNA sequences known to be important in rat growth hormone (rGH) and the human TSH β (hTSH β) gene revealed homology between -210 to -190 of rGH and +23 to +40 of hTSH β . Based on this homology chimeric plasmids containing either -128 to +7 or -128 to +37 of the hTSH β gene fused to the indicator gene, chloramphenicol acetyl transferase (CAT) were constructed yielding pTSH β 128/+7 CAT and pTSH β 128/+37 CAT, respectively. These plasmids also contained the 72 bp repeat enhancer from SV40 because basal expression was quite low without this enhancing element. The plasmids were transfected into both a rGH producing cell line (GH3) or a human embryonal kidney cell line (293). No expression was detected in GH3 cells indicating that either this construct lacked important enhancing elements or contained potent cell specific repressor elements. The latter hypothesis is favored since GH3 cells do not secrete TSH and expression of these plasmids was found in 293 cells.

In 293 cells, these plasmids were employed in T₃ regulatory studies. Under thyroid-free conditions, CAT expression from pTSH β 128/+37CAT was three-fold higher than expression from pTSH β 128/+7CAT. Addition of 10⁻⁸ M T₃, however, resulted in a three-fold decrease in CAT expression from pTSH β 128/+37 CAT but a two-fold increase in CAT expression from pTSH β 128/+7 CAT. CAT expression from either promoterless plasmids or those containing viral promoters was not affected by T₃ treatment. These data indicate that a thyroid hormone inhibitory element (TIE) is located between +8 and +37 in the first exon of the human TSH β gene and suggest that a thyroid hormone stimulatory element (TSE) is located between -128 and +7. The location of a TIE downstream from the start of transcription may explain why T₃ can exert a profound negative control on TSH β gene transcription.

. . . . F. E. Wondisford, B. D. Weintraub

J. Site Directed Mutagenesis of hTSH α and β genes.

Prior studies probing the structure-function correlates of pituitary glycoproteins have provided insight into the importance of certain subunit regions in hormone assembly and function. However, many of these studies have used chemical modifications that may have had other, unintended effect on these hormones. Thus, a process that resulted in specific amino acid changes in the α and β subunits might provide new insights into glycoprotein structure and function. We are currently employing the technique of site directed mutagenesis to investigate the effect of mutations of the hTSH α and β genes on hTSH subunit synthesis, combination, glycosylation, release, receptor binding, and biological activity. Our initial studies have centered on mutations in the glycosylation sites of the α and β subunits. Future studies will include

mutations in areas reportedly involved in subunit combination and receptor binding. After mutation TSH α and β genes will be transfected and transiently expressed into human embryonic kidney cells (293 cells, see below).

. . . . R. W. Lash, F. E. Wondisford, B. D. Weintraub

K. Transfection of hTSH into 293 cells

Previous studies in this laboratory have elucidated the optimal conditions for cotransfection of TSH- α on hTSH- β genes into a eukaryotic cell with subsequent secretion of biologically active TSH. More recent studies have demonstrated hTSH expression in our transient system to be greater than levels seen in stable transfection systems.

We have also observed that that excess concentrations of α subunit may be necessary for driving hTSH subunit combination. Using two different hTSH- β expression vectors, increasing amounts of transfected α subunit resulted in increased amounts of secreted hTSH. Although this relationship has been suspected on the basis of in vitro experiments, this appears to be the first evidence of its existence in vivo.

. . . . R. W. Lash, F. E. Wondisford, B. D. Weintraub

L. Hypothalamic Regulation of TSH Synthesis

The role of hypothalamic TRH in regulating TSH synthesis and secretion in young and old euthyroid and hypothyroid rats was investigated. The hypothyroid rats received either bilateral electrolytic lesions in the paraventricular nuclei (PVN) or similar surgical treatment but had sham lesions placed. After 2 and 4 weeks, anterior pituitaries were analyzed for TSH- β and α mRNA levels by applying samples to Northern gels or slot/blot filters, hybridizing with TSH- β and hCG- α riboprobes, exposure to autoradiograms and quantitated by densitometry. At 2 weeks, TSH- β mRNA levels were increased 5-fold in hypothyroid-sham lesioned rats compared to normal ($p < 0.01$). In contrast, TSH- β mRNA levels were not increased in hypothyroid-PVN lesioned rats as compared to normal. At 4 weeks, TSH- β mRNA were increased 16-fold in hypothyroid-sham lesioned rats ($p < 0.001$) and only 5-fold in hypothyroid-PVN lesioned rats ($p < 0.001$) as compared to normal). α subunit mRNA levels after 4 weeks of hypothyroidism increased 11-fold in the hypothyroid-sham lesioned rats ($p < 0.02$) and did not change in the hypothyroid-PVN lesioned rats as compared to normals. Thus, after 2 and 4 weeks of hypothyroidism, PVN lesions prevented the increase in plasma TSH and pituitary TSH- β and α mRNA levels. Therefore, in hypothyroid rats, factors in the PVN, either directly or indirectly modulate thyroid hormone feedback regulation of TSH subunit synthesis.

. . . . T. Taylor, F. E. Wondisford, B. D. Weintraub

M. Effects of Age on Hypothalamic Regulation

The effects of TRH on the altered TSH synthesis and secretion seen with aging was studied. Rats were thyroidectomized at 7 weeks and studied at 9 weeks and 1 year. TRH mRNA levels in the PVN were measured by in situ

hybridization of brain slices with labeled cDNA probe and autoradiography. Pituitary TSH- β mRNA levels were measured by cytoplasmic RNA hybridization with labeled riboprobe and autoradiography. The the euthyroid state, the old compared to young rats had lower basal free T4 ($p < 0.001$), small but not significant decreases in plasma TSH and pituitary TSH- β mRNA, and similar TRH mRNA levels in the PVN. Acute hypothyroidism for 2 weeks in the young adult rat decreased plasma free T4 ($P < 0.001$), increased plasma TSH 12-fold ($p < 0.001$), TSH- β mRNA 2.3-fold ($p < 0.05$) and TRH mRNA 2.3-fold ($p < 0.05$). In the old rats, chronic hypothyroidism decreased plasma free T4 ($p < 0.001$), increased plasma TSH 25-fold ($p < 0.01$) and TSH mRNA 4-fold ($p < 0.001$), but the old rats demonstrated only a small increase in TRH mRNA of 2.2-fold ($p < 0.05$). In conclusion, hypothalamic regulation of TSH synthesis in response to hypothyroidism is altered with aging and cannot be fully explained by changes in TRH mRNA levels.

. . . . T. Taylor, B. D. Weintraub

N. Endocrine and Development Regulation of TRH Synthesis

The effects of age on the hypothalamic control of thyroid status was studied in euthyroid and hypothyroid perinatal, young adult and 1-year-old rats. The development of TRH gene expression in the rat diencephalon was determined using in situ hybridization histochemistry. The first neurons expressing the TRH gene were found on gestational day 14 (E14) in the lateral hypothalamus, shortly after completion of their last cell division. On E15 and E16, additional labeled cells appeared medially in the dorsomedial and paraventricular nuclei, respectively, followed on E17 by cells in the preoptic area. At birth, TRH mRNA neurons were present in all the locations seen in the adult hypothalamus. Labeling intensity and number of neurons containing TRH mRNA continued to increase throughout this time period. TRH mRNA was not detected in the reticular thalamic nucleus until the 7th postnatal day and progressively increased in intensity of labeling over the next 10 days. On the 21st postnatal day, final adult patterns of expression were present. Thus, the differential expression of TRH mRNA in the hypothalamus and thalamus and the structure-function relationship of the peptide will be interesting to elucidate. In adult rats, it was confirmed that hypothyroidism of 2 weeks duration increased TRH mRNA in the PVN specifically, 3-fold as compared to normal rats ($p < 0.001$) and lesions in the PVN which created hypothyroidism decreased TRH mRNA in the PVN to near undetectable amounts ($p < 0.001$). Thus, age has a significant effect on TRH gene expression in the basal state and in response to hypothyroidism.

. . . . T. Taylor

O. Molecular Basis of Thyroid Hormone Resistance in Man

We have explored the biological function of the c-erbA α and c-erbA β thyroid hormone and receptor genes in patients with generalized thyroid hormone resistance (GTHR) where defective thyroid hormone action is known). We examined the c-erbA α and c-erbA β genes in kindred A with GTHR where a marked decrease in T3-binding affinity of salt-extracted fibroblast nuclear receptors had been demonstrated. Restriction

analysis using the human c-erbA β and c-erbA α cDNA probes failed to identify any abnormal bands on Southern blots. This indicated there were no detectable chromosomal rearrangements or large deletions in these c-erbA genes.

As an alternative method of establishing a relationship between the c-erbA genes and the GTHR trait were performed linkage analysis. We first studied linkage between GTHR and c-erbA β . Seventeen family members of kindred A were typed for polymorphisms of the c-erbA β gene detected with Bam HI and Eco RV. Both RFLPs cosegregated with the GTHR trait, and the kindred was fully informative when family members were haplotyped using the combination of the two RFLPs. Linkage analysis was performed and the maximum lod score between the GTHR and c-erbA β loci was 3.91 at the recombination fraction of 0. The close relationship between a gene that codes for a high-affinity thyroid hormone binding protein and a syndrome with abnormal thyroid hormone action strongly suggests: (1) the c-erbA β gene codes for a physiological thyroid hormone receptor; (2) the syndrome of GTHR in kindred A results from a mutation in the c-erbA β gene.

. . . . S. J. Usala, F. E. Wondisford, R. W. Lash, C. Weinberger, N. Gesundheit, A. E. Bale, B. D. Weintraub

II. HORMONES AND RECEPTORS

A. Insulin-like Growth Factors: Biosynthesis and Action

We have continued our studies of the insulin-like growth factors (IGFs), their receptors and carrier proteins. During the past year we have demonstrated that: (1) multiple IGF-II RNAs (1.2 to 4.6 kb) arise from a single gene through the use of 2 promoters and 2 polyA addition sites; (2) the IGF-II gene is transcribed from both promoters in 11 fetal rat tissues; (3) IGF-II RNA in different tissues is translated into pre-pro-IGF-II is processed to 7.5 kDa IGF-II; (4) the fetal/neonatal form of the IGF carrier protein has been cloned; (5) the carrier protein precursor contains a 34 amino acid prepeptide and the 270 amino acid mature protein; (6) carrier protein mRNA is expressed in multiple fetal tissues, but is decreased in the corresponding adult tissues; (7) polyclonal antibodies to the purified type II IGF receptor do not stimulate or inhibit IGF action in L6 rat myoblasts, suggesting that these effects are mediated by the type I rather than the type II receptor; (8) circulating type II IGF receptors corresponding to the extracellular domain are present in fetal and neonatal rat serum; (9) levels of the circulating type II receptor decrease markedly in older rats; (10) activation of human T lymphocytes results in increased expression of type I and type II IGF receptors, suggesting that the IGFs may participate in the activation cascade; (11) two-chain insulin-IGF hybrid molecules containing the A-domain of IGF-I have increased mitogenic activity and binding to type I IGF receptors but do not bind to IGF carrier proteins.

. . . . M. M. Rechler, A. L. Brown, D. E. Graham, C. C. Orłowski, J.-F. Wang, Y. W.-H Yang, J. A. Romanus, L. Tseng, J. H. Hammond, N.-J. Ge

III. STUDIES OF THE MECHANISM OF THE INSULIN ACTION AND ITS PERTURBATION IN ALTERED METABOLIC STATES

A. Insulin-Cell Interaction

The phosphorylation state and tyrosine kinase activity of insulin receptors in subfractions of insulin-treated rat adipose cells have been studied. The results suggest that insulin receptors retain their kinase activity on internalization, indicating that the receptor kinase may possibly mediate insulin's effects while inside the cell. However, if the internalized receptor kinase mediates insulin's effect on glucose transport, only a portion of its maximum activity appears to be necessary for full glucose transport stimulation (<20%). Further, the difference in kinase activity among subfractions suggests that the receptor kinase in the low-density microsomes may be in the process of deactivation. The effects of isoproterenol and insulin on the subcellular distribution and phosphorylation state of insulin receptors have been investigated in rat adipose cells. The results suggest that isoproterenol augments insulin's effect on receptor internalization, but reverses its stimulatory effect on receptor phosphorylation state and tyrosine kinase activity in plasma membranes; the latter effects may account for the decreased sensitivity of the glucose transport response to insulin.

. . . . T. M. Weber, I. A. Simpson, S. W. Cushman

B. Insulin's Regulation of Glucose Transport

Conditions have been established which allow the isolation of rat adipose cell plasma membranes retaining a large part of the stimulatory effect of insulin in intact cells. Studies with these membranes suggest that in addition to stimulating the translocation of glucose transporters to the plasma membrane, insulin appears to induce a structural or conformational change in the glucose transporter manifested in an altered activation energy for plasma membrane glucose transport and possibly in an altered immunoreactivity as assessed by Western blotting. To examine the possible role of protein kinase C in the signaling mechanism of insulin-stimulated glucose transport in the isolated rat adipose cell, we have compared the effects of insulin and the tumor promoting phorbol ester, phorbol myristate acetate (PMA), on 3-O-methylglucose transport activity and on the distribution of D-glucose-inhibitable cytochalasin B binding sites in rat adipose cells. The data suggest that 1) protein kinase C activation causes the translocation of glucose transporters from an intracellular, low-density microsomal pool to the plasma membrane without a corresponding increase in transport activity and 2) insulin appears to cause the activation of these translocated glucose transporter proteins. We have addressed the question of a long term effect of insulin on adipose cell glucose transporter content by using a cultured cell system, the 3T3-F442A preadipocyte. At confluence, cells were made to differentiate without or with insulin for 15 days. This study, besides documenting the acute effect of insulin on glucose transporter translocation in 3T3-F442A adipose cells, clearly demonstrates that chronic exposure to insulin of these differentiating cells markedly increases the content of glucose transporters. A procedure for purification of the 45 kDa transport protein from rat brain has

been developed. An ~5,000-fold purification of the rat brain glucose transporter has been achieved with a yield of 25%.

. . . . S. W. Cushman, I. A. Simpson, T. M. Weber, M. J. Zarnowski, D. R. Yver, A. D. Habberfield, T. L. Jones, J. Saltis

C. Alterations in Insulin's Action in Insulin-Dependent Diabetes Mellitus

Diabetes and its treatment with insulin in the rat result in dramatic changes in insulin-stimulated glucose transport activity and glucose transporter number in adipose cells. To understand the molecular basis for these changes, we have used the Hep G2 glucose transporter cDNA to assess glucose transporter mRNA transcripts by Northern blotting. The data suggest that 1) glucose transporter number in adipose cells from diabetic rats is not determined by glucose transporter mRNA levels whereas with insulin treatment increased glucose transporter mRNA may be responsible for increased glucose transporter number or 2) the Hep G2 glucose transporter cDNA detects an mRNA which does not encode the major insulin-responsible glucose transporter. Evidence has recently accumulated for a direct role of glucose, independent of insulin, in the regulation of cellular glucose transport. Moreover, we have demonstrated the reversal of in vivo insulin resistance in diabetic rats by normalization of hyperglycemia without any change in plasma insulin concentration. In the present study, the effect of correction of hyperglycemia on insulin's stimulatory action on glucose transport activity in adipose cells from diabetic rats have been examined. The data show that normalization of the plasma glucose concentration in the absence of insulin therapy in diabetic rats restores, or may even enhance, the in vitro adipose cell glucose transport response to insulin while normalizing in vivo insulin-mediated glucose disposal and suggest that the plasma glucose concentration is an important regulator of glucose transport activity in adipose cells, independent of the plasma insulin concentration.

. . . . S. W. Cushman, M. J. Zarnowski, D. R. Yver

D. Insulin's Regulation of Hormone Binding

A comparison of insulin's effects on glucose transport and cell surface IGF-II receptors has been undertaken in rat adipose cells using 3-O-methylglucose transport as a measure of glucose transport activity and Scatchard analysis of IGF-II binding in the presence of KCN to determine cell surface IGF-II receptor number. These results demonstrate that while the characteristics of the stimulatory action of insulin on glucose transport activity and cell surface IGF-II receptor number are qualitatively similar, quantitative differences are clearly demonstrable which suggest that the subcellular cycling of these two integral membrane proteins occurs by distinct processes. The effects of adenosine, isoproterenol, and glucose have now been examined on both steady state insulin responsiveness and sensitivity in this cell type prepared in the presence of saturating adenosine (200 nM). The results show that the

stimulatory effect of insulin on IGF-II binding to rat adipose cells is modulated not only by counterregulatory hormones, but also by glucose, a major substrate of insulin action.

. . . . S. W. Cushman, I. A. Simpson

F. Counterregulation of Insulin's Action by Catecholamines

The counterregulatory action of catecholamines on insulin-stimulated glucose transport and its relation to glucose transporter phosphorylation have been studied in isolated rat adipose cells. The results suggest that the phosphorylation state of the glucose transporter does not appear to be involved in either signaling transporter translocation or triggering changes in transporter intrinsic activity. Insulin shifts the steady state subcellular distribution of IGF-II receptors from a large intracellular pool to the plasma membrane in the rat adipose cell. In the present study, the counterregulatory effects of adrenergic stimulation, adenosine deaminase (ADA), and cAMP on this process have been studied. The results suggest that β -adrenergic stimulation, through a cAMP-dependent mechanism, markedly alters the insulin-stimulated redistribution of IGF-II receptors. This effect is additional to the potent antagonistic action of cAMP on insulin's signaling mechanism.

. . . . I. A. Simpson, T. M. Weber, J. Saltis, S. W. Cushman, M. J. Zarnowski

F. Alterations in Insulin's Action with Fasting/Refeeding

The effects of fasting and refeeding on the glucose transport response to insulin in isolated rat adipose cells have been examined. The results suggest that the insulin resistant glucose transport in isolated adipose cells from fasted rats can be explained by a decreased translocation of glucose transporters to the plasma membrane due to a depleted intracellular pool. In contrast, the insulin hyperresponsive glucose transport observed with refeeding appears to result from 1) a restored translocation of glucose transporters to the plasma membrane from an intracellular pool refilled through an increase in intracellular protein and 2) enhanced plasma membrane glucose transporter intrinsic activity. The human Hep G2 glucose transporter cDNA clone has been used to examine the molecular basis for these alterations. The data suggest that the abundance of mRNAs for multiple adipose cell genes is affected by fasting and refeeding. In particular, this is the first demonstration in an insulin-sensitive tissue that glucose transporter number, and hence a major factor in the glucose transport response to insulin, may be controlled, at least in part, by alterations in mRNA abundance. Insulin increases glucose transport activity and IGF-II binding in rat adipose cells by eliciting the redistribution of glucose transporters and IGF-II receptors from large intracellular pools to the plasma membrane. We now have measured cell surface IGF-II binding in intact cells from 2-day fasted and 2-day fasted/6-day refed rats and assessed IGF-II receptor number in subcellular membrane fractions by immunoblotting. The results suggest that fasting differentially regulates the number and distribution of IGF-II receptors and glucose transporters in adipose cells. This finding suggests distinct

intracellular trafficking pathways for these proteins. Nutritional regulation of the IGF-II receptor may serve as a tool to explore the physiological role of IGF-II.

. . . . S. W. Cushman, I. A. Simpson, M. J. Zarnowski, D. R. Yver

11

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55000-16 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Biosynthesis and Glycosylation of Thyrotropin

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

PI:	B. D. Weintraub	Chief	MCNEB, NIDDK
Others:	N. Gesundheit	Senior Medical Staff Fellow	MCNEB, NIDDK
	P. W. Gyves	Senior Medical Staff Fellow	MCNEB, NIDDK
	T. Taylor	Guest Researcher	MCNEB, NIDDK
	B. S. Stannard	Biologist	MCNEB, NIDDK
	G. S. DeCherney	Guest Researcher	MCNEB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Molecular Regulation and Neuroendocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.3

PROFESSIONAL:

3.3

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

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

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

The role of carbohydrates in most glycoproteins is not known. Moreover, there is little known about the endocrine and developmental regulation of carbohydrate structure. Using newly developed methods of high performance liquid chromatography, we have shown that thyrotropin (TSH) from hypothyroid rodents has more sialylated and less sulfated chains. Similarly, in development there is an increase in sialylation as well as more complex carbohydrate chains. Such changes correlate with a delay in metabolic clearance rate and presumably increased in vivo bioactivity. Inhibitors of carbohydrate processing to final complex sugar structures affect the secretion and sorting of TSH. These studies suggest that carbohydrate processing of glycoproteins may be regulated and affect multiple biological functions.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55001-12 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Regulation and Action of Thyrotropin

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

PI:	B. D. Weintraub	Chief	MCNEB, NIDDK
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	N. R. Thotakura	Visiting Associate	MCNEB, NIDDK
	S. J. Usala	Medical Staff Fellow	MCNEB, NIDDK
	F. E. Wondisford	Medical Staff Fellow	MCNEB, NIDDK
	P. W. Gyves	Guest Researcher	MCNEB, NIDDK

COOPERATING UNITS (if any)

None

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TOTAL MAN-YEARS

3.53

PROFESSIONAL:

3.53

OTHER:

0

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- (a1) Minors
- (a2) Interviews

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

The human thyrotropin (TSH) receptor has not previously been purified or characterized. This receptor is not only important for its interaction with thyrotropin, but also for its interaction with thyroid-stimulating antibodies in Graves' Disease (a major cause of hyperthyroidism), as well as thyroid-blocking antibodies in idiopathic hypothyroidism. Using recombinant DNA methodology and monoclonal antibodies to the TSH receptor we have isolated several cDNA clones as well as 2 variant genes coding for a TSH receptor. This receptor shows specific binding to TSH as well as both stimulating and blocking antibodies. It has 7 membrane spanning domains analogous to several other polypeptide hormone receptors coupled to guanine nucleotide binding proteins.

Enzymatic deglycosylation of TSH has been achieved by newly available endoglycosidase F preparations. The deglycosylated molecule shows decreased in vivo and in vitro bioactivity.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55002-08 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular Biology of Glycoprotein Hormones

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

PI:	B. D. Weintraub	Chief	MCNEB, NIDDK
Others:	F. E. Wondisford	Medical Staff Fellow	MCNEB, NIDDK
	S. Usala	Medical Staff Fellow	MCNEB, NIDDK
	J. M. Moates	Guest Researcher	MCNEB, NIDDK
	T. Taylor	Guest Researcher	MCNEB, NIDDK
	R. W. Lash	Medical Staff Fellow	MCNEB, NIDDK
	A. E. Bale	Biotechnology Fellow	DCBD, NCI
	C. Weinberger	Senior Staff Fellow	LCB, NIMH

COOPERATING UNITS (if any)

None

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TOTAL MAN-YEARS

3.7

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

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

The human thyrotropin (TSH) beta subunit gene has been isolated and sequenced. It differs from previously isolated rodent TSH-beta genes in promotor organization, which may have important implications for promotor function in man. The gene has been expressed in mammalian cells, and by co-transfection with excess alpha subunit cDNA, biologically active thyrotropin has been produced. Currently we are studying regulatory elements in the promotor region of the gene to understand the control of TSH production in man. Similarly, through site directed mutagenesis we are exploring structure-function relationships of the coding regions of the gene.

Hypothalamic regulation of TSH is being studied by examining TRH messenger RNA in rodents of various ages and different endocrine status. We have observed that hypothalamic factors are important in the pituitary response to hypothyroidism.

The syndrome of generalized thyroid hormone resistance has been linked to the human c-erbA beta gene by restriction fragment length polymorphism analysis. This implies that the beta gene codes for a receptor important for thyroid hormone action in man.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55003-15 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

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

Molecular Mechanisms in Neuroendocrine Peptide and Protein Pathways

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

P.I.:	Irwin M. Chaiken	Research Chemist	MCNEB, NIDDK
Others:	Shoji Ando	Visiting Fellow	MCNEB, NIDDK
	Giorgio Fassina	Visiting Fellow	MCNEB, NIDDK

COOPERATING UNITS (if any)

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Laboratory of Biochemistry and Metabolism, NIDDK

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PROFESSIONAL:

OTHER:

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

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

Terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55004-18 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanisms of Peptide and Protein Recognition, Assembly, Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	Irwin M. Chaiken	Research Chemist	MCNEB, NIDDK
Others:	Giorgio Fassina	Visiting Fellow	MCNEB, NIDDK
	Shoji Ando	Visiting Fellow	MCNEB, NIDDK
	Yechiel Shai	Guest Researcher	MCNEB, NIDDK

COOPERATING UNITS (if any)

Inst. of Organic Chem., Univ. of Padova, Italy; Biophysics Dept., Johns Hopkins Medical School, Baltimore, MD

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TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

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- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55005-18 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Biorecognition Technology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Irwin M. Chaiken	Research Chemist	MCNEB, NIDDK
Others:	Giorgio Fassina	Visiting Fellow	MCNEB, NIDDK
	Yechiel Shai	Guest Researcher	MCNEB, NIDDK
	Paolo Caliceti	Guest Researcher	MCNEB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Molecular Regulation and Neuroendocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55006-15 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Insulin-like Growth Factors: Biosynthesis and Action

PRINCIPLE INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	M.M. Rechler	Chief, GD Section	MCNEB, NIDDK	J.H. Hammond	Special Volunteer
Others:	A.L. Brown	Staff Fellow	MCNEB, NIDDK	N.-J. Ge	Special Volunteer
	D.E. Graham	Expert	MCNEB, NIDDK		
	C.C. Orłowski	Staff Fellow	MCNEB, NIDDK		
	J.-F. Wang	Visiting Fellow	MCNEB, NIDDK		
	Y.W.-H. Yang	Staff Fellow	MCNEB, NIDDK		
	J.A. Romanus	Biologist	MCNEB, NIDDK		
	L. Tseng	Chemist	MCNEB, NIDDK		

COOPERATING UNITS (if any)

MB NCI (S.P. Nissley, W. Kiess); Univ. of Naples, Italy (C.B. Bruni, R. Frunzio, L. Chiariotti); Mt. Sinai Sch. Med., CUNY, NY, (G.T. Burke, P.G. Katsoyannis)

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Growth and Development Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

9.25

PROFESSIONAL:

6.75

OTHER:

2.5

CHECK APPROPRIATE BOX(ES)

(a) Human subjects (b) Human tissues (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

We have continued our studies of the insulin-like growth factors (IGFs), their receptors and carrier proteins. During the past year we have demonstrated that: (1) multiple IGF-II RNAs (1.2 to 4.6 kb) arise from a single gene through the use of 2 promoters and 2 polyA addition sites; (2) the IGF-II gene is transcribed from both promoters in 11 fetal rat tissues; (3) IGF-II RNA in different tissues is translated into pre-pro-rIGF-II is processed to 7.5 kDa IGF-II; (4) the fetal/neonatal form of the IGF carrier protein has been cloned; (5) the carrier protein precursor contains a 34 amino acid prepeptide and the 270 amino acid mature protein; (6) carrier protein mRNA is expressed in multiple fetal tissues, but is decreased in the corresponding adult tissues; (7) polyclonal antibodies to the purified type II IGF receptor do not stimulate or inhibit IGF actions in L6 rat myoblasts, suggesting that these effects are mediated by the type I rather than the type II receptor; (8) circulating type II IGF receptors corresponding to the extracellular domain are present in fetal and neonatal rat serum; (9) levels of the circulating type II receptor decrease markedly in older rats; (10) activation of human T lymphocytes results in increased expression of type I and type II IGF receptors, suggesting that the IGFs may participate in the activation cascade; (11) two-chain insulin-IGF hybrid molecules containing the A-domain of IGF-I have increased mitogenic activity and binding to type I IGF receptors but do not bind to IGF carrier proteins.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 55007-10 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin-Cell Interaction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: T. M. Weber Staff Fellow MCNEB, NIDDK

Others: I. A. Simpson Visiting Scientist MCNEB, NIDDK
S. W. Cushman Chief, EDMNS MCNEB, NIDDK

COOPERATING UNITS (if any) Department of Medicine, University of Bari, Bari, Italy (S. DiPaolo); Institute of Pharmacology and Toxicology, University of Göttingen, Göttingen, Germany (H. G. Joost); Fermentation Research Laboratories, Sankyo Company, Tokyo, Japan (M. Kuroda).

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.9

PROFESSIONAL:

0.9

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The phosphorylation state and tyrosine kinase activity of insulin receptors in subfractions of insulin-treated rat adipose cells have been studied. The results suggest that insulin receptors retain their kinase activity on internalization, indicating that the receptor kinase may possibly mediate insulin's effects while inside the cell. However, if the internalized receptor kinase mediates insulin's effect on glucose transport, only a portion of its maximum activity appears to be necessary for full transport stimulation (<20%). Further, the difference in kinase activity among subfractions suggests that the receptor kinase in the low-density microsomes may be in the process of deactivation. The effects of isoproterenol and insulin on the subcellular distribution and phosphorylation state of insulin receptors have been investigated in rat adipose cells. The results suggest that isoproterenol augments insulin's effect on receptor internalization, but reverses its stimulatory effect on receptor phosphorylation state and tyrosine kinase activity in plasma membranes; the latter effects may account for the decreased sensitivity of the glucose transport response to insulin.

11
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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55008-10 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Insulin's Regulation of Glucose Transport

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal investigator) (Name, title, laboratory, and institute affiliation)

PI:	S. W. Cushman	Chief, EDMS	MCNEB, NIDDK
Others:	I. A. Simpson	Visiting Scientist	MCNEB, NIDDK
	T. M. Weber	Staff Fellow	MCNEB, NIDDK
	M. J. Zarnowski	Biologist	MCNEB, NIDDK
	D. R. Yver	Chemist	MCNEB, NIDDK
	A. D. Habberfield	Visiting Fellow	MCNEB, NIDDK
	T. L. Jones	Medical Staff Fellow	MCNEB, NIDDK
	J. Saltis	Visiting Fellow	MCNEB, NIDDK

COOPERATING UNITS (Name)	Special volunteer	MCNEB, NIDDK
--------------------------	-------------------	--------------

Institute of Pharmacology and Toxicology, University of Göttingen, Göttingen, Germany (H. G. Joost); INSERM Unit 177, Paris, France (M. Lavau, M. Guerre-Millo).

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

6.0

PROFESSIONAL

6.0

OTHER

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Conditions have been established which allow the isolation of rat adipose cell plasma membranes retaining a large part of the stimulatory effect of insulin in intact cells. Studies with these membranes suggest that in addition to stimulating the translocation of glucose transporters to the plasma membrane, insulin appears to induce a structural or conformational change in the glucose transporter manifested in an altered activation energy for plasma membrane glucose transport and possibly in an altered immunoreactivity as assessed by Western blotting. To examine the possible role of protein kinase C in the signaling mechanism of insulin-stimulated glucose transport in the isolated rat adipose cell, we have compared the effects of insulin and the tumor promoting phorbol ester, phorbol myristate acetate (PMA), on 3-O-methylglucose transport activity and on the distribution of D-glucose-inhibitable cytochalasin B binding sites in rat adipose cells. The data suggest that 1) protein kinase C activation causes the translocation of glucose transporters from an intracellular, low-density microsomal pool to the plasma membrane without a corresponding increase in transport activity and 2) insulin appears to cause the activation of these translocated glucose transporter proteins. We have addressed the question of a long term effect of insulin on adipose cell glucose transporter content by using a cultured cell system, the 3T3-F442A preadipocyte. At confluence, cells were made to differentiate without or with insulin for 15 days. This study, besides documenting the acute effect of insulin on glucose transporter translocation in 3T3-F442A adipose cells, clearly demonstrates that chronic exposure to insulin of these differentiating cells markedly increases the content of glucose transporters. A procedure for purification of the 45 kDa transport protein from rat brain has been developed. An $\approx 5,000$ -fold purification of the rat brain glucose transporter has been achieved with a yield of 25%.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55010-07 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Insulin's Action in Insulin-Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	S. W. Cushman	Chief, EDMNS	MCNEB, NIDDK
Others:	M. J. Zarnowski	Biologist	MCNEB, NIDDK
	D. R. Yver	Chemist	MCNEB, NIDDK

COOPERATING UNITS (if any)

Diabetes Unit, Beth Israel Hospital, Boston, MA (B. B. Kahn, J. S. Flier);
 Department of Medicine, Yale University, New Haven, CN (L. Rossetti, R. A.
 DeFronzo).

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.7

PROFESSIONAL

0.7

OTHER:

0.0

CHECK APPROPRIATE BOXES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Diabetes and its treatment with insulin in the rat result in dramatic changes in insulin-stimulated glucose transport activity and glucose transporter number in adipose cells. To understand the molecular basis for these changes, we have used the Hep G2 glucose transporter cDNA to assess glucose transporter mRNA transcripts by Northern blotting. The data suggest that 1) glucose transporter number in adipose cells from diabetic rats is not determined by glucose transporter mRNA levels whereas with insulin treatment increased glucose transporter mRNA may be responsible for increased glucose transporter number or 2) the Hep G2 glucose transporter cDNA detects an mRNA which does not encode the major insulin-responsive glucose transporter. Evidence has recently accumulated for a direct role of glucose, independent of insulin, in the regulation of cellular glucose transport. Moreover, we have demonstrated the reversal of *in vivo* insulin resistance in diabetic rats by normalization of hyperglycemia without any change in plasma insulin concentration. In the present study, the effect of correction of hyperglycemia on insulin's stimulatory action on glucose transport activity in adipose cells from diabetic rats has been examined. The data show that normalization of the plasma glucose concentration in the absence of insulin therapy in diabetic rats restores, or may even enhance, the *in vitro* adipose cell glucose transport response to insulin while normalizing *in vivo* insulin-mediated glucose disposal and suggest that the plasma glucose concentration is an important regulator of glucose transport activity in adipose cells, independent of the plasma insulin concentration.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55011-06 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Insulin's Action with Chronic Hyperinsulinemia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: S. W. Cushman Chief, EDMNS MCNEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Inactive.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55012-06 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin's Regulation of Hormone Binding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: S. W. Cushman Chief, EDMNS MCNEB, NIDDK

Others: I. A. Simpson Visiting Scientist MCNEB, NIDDK

COOPERATING UNITS (if any)

Research Laboratories, A. H. Robins Company, Richmond, VA (K. C. Appell).

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A comparison of insulin's effects on glucose transport and cell surface IGF-II receptors has been undertaken in rat adipose cells using 3-O-methylglucose transport as a measure of glucose transport activity and Scatchard analysis of IGF-II binding in the presence of KCN to determine cell surface IGF-II receptor number. The results demonstrate that while the characteristics of the stimulatory action of insulin on glucose transport activity and cell surface IGF-II receptor number are qualitatively similar, quantitative differences are clearly demonstrable which suggest that the subcellular cycling of these two integral membrane proteins occurs by distinct processes. The effects of adenosine, isoproterenol, and glucose have now been examined on both steady state insulin responsiveness and sensitivity in this cell type prepared in the presence of saturating adenosine (200 nM). The results show that the stimulatory effect of insulin on IGF-II binding to rat adipose cells is modulated not only by counterregulatory hormones, but also by glucose, a major substrate of insulin action.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55013-05 MCNEB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Counterregulation of Insulin's Action by Catecholamines

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: I. A. Simpson Visiting Scientist MCNEB, NIDDK

Others: T. M. Weber Staff Fellow MCNEB, NIDDK

J. Saltis Visiting Fellow MCNEB, NIDDK

S. W. Cushman Chief, EDMNS MCNEB, NIDDK

M. J. Zarnowski Biologist MCNEB, NIDDK

COOPERATING UNITS (if any) Institute of Pharmacology and Toxicology, University of Göttingen, Göttingen, Germany (H. G. Joost); Department of Medicine, University of Gothenburg, Gothenburg, Sweden (P. N. Lönnroth, U. Smith); Research Laboratories, A. H. Robins Company, Richmond, VA (K. C. Appell).

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.3

PROFESSIONAL

1.3

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The counterregulatory action of catecholamines on insulin-stimulated glucose transport and its relation to glucose transporter phosphorylation have been studied in isolated rat adipose cells. The results suggest that the phosphorylation state of the glucose transporter does not appear to be involved in either signaling transporter translocation or triggering changes in transporter intrinsic activity. Insulin shifts the steady state subcellular distribution of IGF-II receptors from a large intracellular pool to the plasma membrane in the rat adipose cell. In the present study, the counterregulatory effects of adrenergic stimulation, adenosine deaminase (ADA), and cAMP on this process have been studied. The results suggest that β -adrenergic stimulation, through a cAMP-dependent mechanism, markedly alters the insulin-stimulated redistribution of IGF-II receptors. This effect is additional to the potent antagonistic action of cAMP on insulin's signaling mechanism.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55014-05 MCNE

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Alterations in Insulin's Action with Fasting/Refeeding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	S. W. Cushman	Chief, EDMNS	MCNEB, NIDDK
Others:	I. A. Simpson	Visiting Scientist	MCNEB, NIDDK
	M. J. Zarnowski	Biologist	MCNEB, NIDDK
	D. R. Yver	Chemist	MCNEB, NIDDK

COOPERATING UNITS (if any)

Diabetes Unit, Beth Israel Hospital, Boston, MA (B. B. Kahn, J. S. Flier);
 Research Laboratories, A. H. Robins Company, Richmond, VA (K. C. Appell).

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

0.7

PROFESSIONAL:

0.7

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The effects of fasting and refeeding on the glucose transport response to insulin in isolated rat adipose cells have been examined. The results suggest that the insulin resistant glucose transport in isolated adipose cells from fasted rats can be explained by a decreased translocation of glucose transporters to the plasma membrane due to a depleted intracellular pool. In contrast, the insulin hyperresponsive glucose transport observed with refeeding appears to result from 1) a restored translocation of glucose transporters to the plasma membrane from an intracellular pool replenished through an increase in intracellular protein and 2) enhanced plasma membrane glucose transporter intrinsic activity. The human Hep G2 glucose transporter cDNA clone has been used to examine the molecular basis for these alterations. The data suggest that the abundance of mRNAs for multiple adipose cell genes is affected by fasting and refeeding. In particular, this is the first demonstration in an insulin-sensitive tissue that glucose transporter number, and hence a major factor in the glucose transport response to insulin, may be controlled, at least in part, by alterations in mRNA abundance. Insulin increases glucose transport activity and IGF-II binding in rat adipose cells by eliciting the redistribution of glucose transporters and IGF-II receptors from large intracellular pools to the plasma membrane. We now have measured cell surface IGF-II binding in intact cells from 2-day fasted and 2-day fasted/6-day refed rats and assessed IGF-II receptor number in subcellular membrane fractions by immunoblotting. The results suggest that fasting differentially regulates the number and distribution of IGF-II receptors and glucose transporters in adipose cells. This finding suggests distinct intracellular trafficking pathways for these proteins. Nutritional regulation of the IGF-II receptor may serve as a tool to explore the physiological role of IGF-II.

ANNUAL REPORT OF THE LABORATORY OF STRUCTURAL BIOLOGY
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

I. BIOLOGY OF COMPLEX CARBOHYDRATES

The cell surface receptor for many pulmonary pathogenic bacteria has been identified as the carbohydrate sequence GalNAc β 1-4Gal β 1-... Sialylated derivatives of this sequence are not receptors suggesting a biochemical basis for the relationship between viral and bacterial pneumonia: viral neuraminidase may desialylate carbohydrate sequences on the cells lining the respiratory tract and increase the number of structures containing unsubstituted GalNAc β 1-4Gal residues which serve as receptors for the pathogenic bacteria. *Escherichia coli* K-99 is responsible for piglet diarrhea. The receptor for this organism has been isolated from piglet intestine and identified as the ganglioside N-glycolyl GM3 (NeuGc α 2-3Gal β 1-4Glc β 1-1Cer). Monoclonal antibody AA4 inhibits the binding of IgE molecules to surface receptors on basophils and mast cells. A glycolipid antigen for the antibody has been isolated and identified as a novel ganglioside with the following structure: Gal α 1-3Gal β 1-3GalNAc β 1-4[NeuAc α 2-8NeuAc α 2-3]Gal β 1-4Glc β 1-1Cer.

.....Drs. V. Ginsburg, N.H Guo, L. Zhang, M. Kyogashima, H. Krivan
D. D. Roberts

II. METABOLISM AND ROLE OF POLYSACCHARIDE SULFATES

The discovery of a novel sulfatase of unusual specificity and the definitive synthesis of isomeric glucosamine sulfates have led to the discovery that heparin contains a unique 3-O,N disulfated glucosamine residue which is essential for its role as an anticoagulant. The enzyme has been partially purified from human urine. The effort to achieve a high level of enzyme purification by means of affinity adsorbants is continuing.

Many polyanions, including heparin, bind to hemoglobin and markedly affect its solubility. In a study of the effects of polyanions of controlled size, highly sulfated trehalose and stachyose have been prepared and tested for their ability to induce allosteric changes in sickle hemoglobin. Both compounds bind with high affinity to hemoglobin-S and strongly decrease its affinity for oxygen. Sulfated trehalose increased the solubility of hemoglobin S. Studies of the effects of other highly sulfated anions, of appropriate dimensions, on the solubility of hemoglobin-S are being carried out.

.....Dr. I. G. Leder

III. EXPRESSION AND FUNCTION OF BACTERIAL CELL SURFACE COMPONENTS

The Vi-antigen of Salmonella typhi is a surface polysaccharide capsule that participates in the protection of the cell from host defense mechanisms and thus increases virulence. We have shown that the Vi-antigen is not involved in protection of the cell against serum-killing but does exert an antiphagocytic effect by interfering with the opsonic effect of specific antibody, mediated through alternate pathway activation of complement. In the presence of normal or C4D serum, strains that differed only in the presence or absence of the Vi-antigen both bound and consumed equivalent amounts of C3. The addition of Vi-specific antibody increased both the rate and extent of phagocytosis by J774 macrophages without altering the amount of bound C3.

Gram-negative bacteria grown in media containing 1-5 mM sodium salicylate or aspirin become phenotypically resistant to various antibiotics including beta-lactams. This increased resistance requires protein synthesis for expression and is due to a 70-90% decrease in the permeation of these antibiotics through the outer membrane barrier. The antibiotic permeation begins to decrease within 5 minutes after the addition of salicylate to the growth medium and reaches a minimum before 2 hours. Growth in salicylate eliminates, or dramatically reduces, the level of the OmpF porin in the outer membrane. When bacteria are grown in salicylate, ompF translation decreases 40 fold whereas ompF transcription is relatively unaffected. These effects may be explained by an increased level of micF RNA in salicylate grown cells. This RNA is complementary to and binds directly to the 5 end of ompF RNA, and inhibits translation.

The concentrations of salicylate used in these studies approximates those achieved in the serum of patients treated with high doses of aspirin and resistance to the antibiotics studied exceed the serum levels of antibiotics commonly achieved during therapy.

.....Drs. J. Foulds, V. Jimenez

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57000-23 LSB

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Complex Carbohydrates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Victor Ginsburg, Ph.D.	Chief, LSB	LSB	NIDDK
Others:	Neng Hua Guo, M.D.	Visiting Fellow	LSB	NIDDK
	Lijuan Zhang	Visiting Fellow	LSB	NCI
	Mamoru Kyogashima, Ph.D., M.D.	Visiting Fellow	LSB	NIDDK
	Howard Krivan, Ph.D.	Staff Fellow	LSB	NIDDK
	David Roberts, Ph.D.	Sr. Staff Fellow	LSB	NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Structural Biology

SECTION

Section on Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

7

PROFESSIONAL

6

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The cell surface receptor for many pulmonary pathogenic bacteria has been identified as the carbohydrate sequence GalNac β 1-4Gal β 1-... Sialylated derivatives of this sequence are not receptors suggesting a biochemical basis for the relationship between viral and bacterial pneumonia: viral neuraminidase may desialylate carbohydrate sequences on the cells lining the respiratory tract and increase the number of structures containing unsubstituted GalNac β 1-4Gal residues which serve as receptors for the pathogenic bacteria.

Escherichia coli K-99 is responsible for piglet diarrhea. The receptor for this organism has been isolated from piglet intestine and identified as the ganglioside N-glycolyl GM3 (NeuGac2-3Gal β 1-4Glc β 1-1Cer).

Monoclonal antibody AA4 inhibits the binding of IgE molecules to surface receptors on basophils and mast cells. A glycolipid antigen for the antibody has been isolated and identified as a novel ganglioside with the following structure:

Gal α 1-3Gal β 1-3GalNac β 1-4[NeuAc α 2-8NeuAc α 2-3]Gal β 1-4Glc β 1-1Cer.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DK 57001-11 LSB

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Metabolism and Role of Polysaccharide Sulfates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Irwin G. Leder Research Chemist LSB NIDDK

COOPERATING UNITS (if any)

Allen Minton, LBP, NIDDK
William Poillon, Center for Sickle Cell Disease, Howard Univ.

LAB/BRANCH

Laboratory of Structural Biology

SECTION

Section on Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1

PROFESSIONAL

1

OTHER

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided)

The discovery of a novel sulfatase of unusual specificity and the definitive synthesis of isomeric glucosamine sulfates have led to the discovery that heparin contains a unique 3-0,N disulfated glucosamine residue which is essential for its role as an anticoagulant. The enzyme has been partially purified from human urine. The effort to achieve a high level of enzyme purification by means of affinity adsorbants is continuing.

Many polyanions, including heparin, bind to hemoglobin and markedly affect its solubility. In a study of the effects of polyanions of controlled size, highly sulfated trehalose and stachyose have been prepared and tested for their ability to induce allosteric changes in sickle hemoglobin. Both compounds bind with high affinity to hemoglobin-S and strongly decrease its affinity for oxygen. Sulfated trehalose increased the solubility of hemoglobin S. Studies of the effects of other highly sulfated anions, of appropriate dimensions, on the solubility of hemoglobin-S are being carried out.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Expression and Function of Bacterial Cell Surface Components

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: John Foulds Research Biochemist LSB NIDDK

Others: Victor Jimenez, M.D., M. Sc. Staff Fellow LSB NIDDKK

COOPERATING UNITS (if any)

Judeh Rosner, LMB, NIDDK
Maureen McKenzie, D, NIDDK

LAB/BRANCH

Laboratory of Structural Biology

SECTION

Section on Membrane Biology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.0

PROFESSIONAL:

2.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Vi-antigen of Salmonella typhi is a surface polysaccharide capsule that participates in the protection of the cell from host defense mechanisms and thus increases virulence. We have shown that the Vi-antigen is not involved in protection of the cell against serum-killing but does exert an antiphagocytic effect by interfering with the opsonic effect of specific antibody, mediated through alternate pathway activation of complement. In the presence of normal or C4D serum, strains that differed only in the presence or absence of the Vi-antigen both bound and consumed equivalent amounts of C3. The addition of Vi-specific antibody increased both the rate and extent of phagocytosis by J774 macrophages without altering the amount of bound C3.

Gram-negative bacteria grown in media containing 1-5 mM sodium salicylate or aspirin become phenotypically resistant to various antibiotics including beta-lactams. This increased resistance requires protein synthesis for expression and is due to a 70-90% decrease in the permeation of these antibiotics through the outer membrane barrier. The antibiotic permeation begins to decrease within 5 minutes after the addition of salicylate to the growth medium and reaches a minimum before 2 hours. Growth in salicylate eliminates, or dramatically reduces, the level of the ompF porin in the outer membrane. When bacteria are grown in salicylate, ompF translation decreases 40 fold whereas ompF transcription is relatively unaffected. These effects may be explained by an increased level of micF RNA in salicylate grown cells. This RNA is complementary to and binds directly to the 5' end of ompF RNA, and inhibits translation.

The concentrations of salicylate used in these studies approximates those achieved in the serum of patients treated with high doses of aspirin and resistance to the antibiotics studied exceed the serum levels of antibiotics commonly achieved during therapy.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 57003-02 LSB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Function of Complex Carbohydrates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: John L. Magnani, Ph. D. Research Chemist LSB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Structural Biology

SECTION

Section on Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Md. 20892

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

1000

ANNUAL REPORT
THE LABORATORY OF MOLECULAR AND CELLULAR BIOLOGY

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The LMCB comprises several groups. One group, led by Dr. Carter, studies gene regulation in mammalian cell systems and is particularly interested in developing efficient vector systems for delivery of genes into cells. The second group, led by Dr. Oka, is generally interested in the endocrine control of differentiation of the mouse mammary gland and has focused on physiological effects of EGF and the molecular biology of various genes which are important in this process. A third project led by Dr. Tietze, is aimed at understanding the molecular basis of several human genetic defects which result in lysosomal storage diseases. This project is conducted in collaboration with workers in NICHD. In addition Dr. Carter's group has initiated a new project as part of the NIH Intramural AIDS Targeted Antiviral Program.

Function of DNA Virus Genomes in Animal Cells

The group led by B. Carter has continued to employ DNA viruses as molecular probes to study genome expression in human cells. We are studying intensively the structure and function of adeno-associated virus (AAV) since this virus has only a small genome. AAV has also been developed as a eukaryotic expression vector. AAV normally grows in cells only in the presence of a helper virus (either adenovirus or herpesvirus). In the absence of any helper the AAV genome integrates into the cell chromosome. Thus, the AAV vector is useful as a transducing virus for high frequency integration of genes in mammalian cell chromosomes to yield stable expression. This vector also may be useful for therapy. Award of a patent for this vector system is imminent. We are now analyzing intensively the control of gene regulation in AAV vectors in order to maximize the expression of foreign genes introduced into mammalian cells using this vector. We have discovered a complex system of gene regulation mediated by products of the AAV rep gene which are required for replication of AAV DNA but also mediate transcriptional activation and also translational inhibition of some genes. We have recently developed extensive use of site-directed mutagenesis to resolve the functions of the rep gene. Coding of all these functions in a single gene appears to be unique in eukaryotic systems. We are also studying adenovirus since this is the helper virus for AAV multiplication. This helper relationship is being analyzed. Also, both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus, this causes malignant transformation of the cell. AAV inhibits this transformation and also inhibits Ad12 oncogenesis in newborn animals. The mechanism of this inhibition of tumor induction by AAV is being studied at the molecular level in both cell culture and in animal experiments. We are also studying mutations in mouse 3T3 cells which render the cells resistant to malignant transformation by a single oncogene (ras) but allow malignant transformation by two oncogenes (ras, myc) acting in concert.

Hormonal Regulation of Cell Growth and Differentiation

Epidermal growth factor (EGF) is produced in large amounts by the mouse submandibular gland. It is also present in such biological fluids as plasma,

milk, urine and saliva. EGF is a potent mitogen for a wide variety of cells in culture but its function in the body needs to be elucidated. Dr. Oka's studies have demonstrated that EGF plays a key role in the development of the mammary gland during pregnancy and mammary tumorigenesis in female mice; in males it serves a role in spermatogenesis by stimulating the meiosis of spermatocytes. Studies have been continued to elucidate the physiological role of EGF by employing a variety of experimental approaches, including radioimmunoassay of EGF in tissues and biological fluids, EGF receptor assay and bioassay of EGF in cell culture. In addition, a useful means of causing EGF deficiency in mice by removal of the submandibular gland and/or administration of anti-EGF antiserum has been established. These procedures, combined with EGF replacement therapy have provided valuable information concerning the function of EGF in the body. These studies have shown that the concentration of EGF in the submandibular gland and plasma of female mice increases significantly during pregnancy. Attenuation of the rise in EGF by sialoadenectomy and anti-EGF treatment resulted in increased rate of spontaneous abortion, suggesting that EGF is necessary for the normal course of pregnancy. In addition, EGF has been shown to have a physiological role in maintaining the normal structure of the epidermis. Additional studies also have revealed that milk contains a high concentration of EGF which serves a physiological function by promoting neonatal eyelid opening.

Lysosomal Transport and Storage Disease

This work is being conducted by Dr. Frank Tietze. Degradation of cellular biopolymers such as proteins and polysaccharides takes place chiefly within the lysosome. The end-products of this degradation, viz., amino acids and monosaccharides, are presumed to exit the lysosome to the cytoplasm, where further metabolism or expulsion to the external medium occurs. To study the process of lysosomal transport, methods were developed to load lysosomes of various cells with amino acids (e.g., cystine, tyrosine) or with a specific monosaccharide (viz., sialic acid) and to measure their rates of egress from the organelle. Studies of cystine egress from lysosomes of human polymorphonuclear leukocytes and of tyrosine from cultured rat thyroid cell lysosomes have revealed these processes to be carrier-mediated and stereo-specific. The further demonstration that no egress of cystine could be detected from similarly loaded lysosomes from patients with the inherited disorder cytinosis indicated that this storage disease is due to a congenital defect of a specific lysosomal carrier. Similar studies on the egress of sialic acid from fibroblast lysosomes have suggested strongly that impaired lysosomal transport underlies another lysosomal storage disorder, free sialic acid storage disease. In addition to a carrier system specific for the lysosomal transport of tyrosine, preliminary evidence has indicated that lysosomes from cultured rat thyroid cells also possess a carrier for moniodotyrosine an end-product of the lysosomal catabolism of thyroglobulin.

Regulation of HIV by AAV

This is a new project aimed at developing possible alternate and novel antiviral therapies for AIDS. Because current work suggests that production of vaccines or use of nucleotide analog therapies for AIDS may be limited and difficult other therapeutic approaches are urgently required. We are attempting to develop a novel approach by using the negative regulatory property of a trans-acting gene of the human parvovirus, AAV, to inhibit the

function of the trans-acting gene tat, of HIV. A functional tat gene is required for HIV growth so inhibition of tat presents a potentially useful approach to an antiviral therapy.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 57501-12 LMCB

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Function of DNA Virus Genomes in Animal Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.: Barrie J. Carter Chief, LMCB LMCB:NIDDK

Other: Ella Mendelson	Visiting Scientist	LMCB:NIDDK
Christeine Lally	Visiting Fellow	LMCB:NIDDK
James Trempe	Senior Staff Fellow	LMCB:NIDDK
Nor Chejanovsky	Visiting Associate	LMCB:NIDDK
Irving Miller	Biologist	LMCB:NIDDK
Brunhild Redemann	Guest Worker	LMCB:NIDDK

COOPERATING UNITS (if any)

V. Nikodem, S. Usala CEB, NIDDK; B. Weintraub, F. Wondisford, MCEB, NIDDK
M.G. Smith, Univ. Otago, New Zealand; J. Tal, Beersheba, Israel

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

7.1

PROFESSIONAL

6.1

OTHER

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are employing DNA viruses as molecular probes to study genome expression in human cells. We are studying intensively the structure and function of a human parvovirus, adeno-associated virus (AAV) since this virus has only a small genome. AAV has been developed as a eukaryotic expression vector. AAV normally grows in cells only in the presence of a helper virus (either adenovirus or herpesvirus). In the absence of any helper, the AAV genome integrates into the cell chromosome. Thus, the AAV vector is useful as a transducing virus for high frequency integration of genes into mammalian cell chromosomes to yield stable expression. This vector also may be useful for gene therapy. We are now analyzing intensely the control of gene regulation in AAV vectors in order to maximize the expression of foreign genes introduced into mammalian cells using this vector. We have discovered a complex system of gene regulation mediated by products of the AAV rep gene which are required for replication of AAV DNA but also mediate transcriptional activation and translational inhibition of some genes. Site-specific mutagenesis and being used to reduce these functions. Coding of all these functions in a single gene is unique in eukaryotic systems. Adenovirus is the helper for AAV. This relationship is being analyzed. Both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus this causes malignant transformation of the cell. AAV inhibits this transformation and also inhibits Ad12 oncogenesis in newborn animals. Thus, AAV inhibits tumor induction. The mechanism of this inhibition of tumor induction is being studied at the molecular level in cell culture. We have recently established a project to analyze interactions of AAV with HIV as a potential approach to a novel therapy for AIDS.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 57502-15 LMCB

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hormonal Regulation of Cell Growth and Differentiation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Takami Oka	Senior Investigator	LMCB:NIDDK
Other:	Flavia Borellini	Visiting Fellow	LMCB:NIDDK
	Ryuhei Kanamoto	Guest Worker	LMCB:NIDDK
	Soji Kasayama	Visiting Fellow	LMCB:NIDDK
	John W. Perry	Biologist	LMCB:NIDDK
	Katsuya Wada	Guest Worker	LMCB:NIDDK
	Masami Yoshimura	Visiting Associate	LMCB:NIDDK

COOPERATING UNITS (if any)

Dr. Shinichi Hayashi, Jikei Medical University, Japan
 Dr. Charles Edwards, LMFG, NIDDK

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

7.0

PROFESSIONAL:

6.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Epidermal growth factor (EGF) exerts diverse biological actions, influencing proliferation, differentiation and metabolic activities of various types of mammalian cells. EGF is produced in large amounts by the mouse submandibular gland and it is also present in various biological fluids such as plasma, milk, urine and saliva. Previously we have presented several lines of evidence indicating that EGF plays an important physiological function in reproductive processes such as maintenance of normal pregnancy, development of mammary gland and spermatogenesis. In addition, EGF has been shown to serve as a growth promoter of mammary tumors. We have also shown that EGF is necessary for maintaining the normal epidermal structure and promoting neonatal eyelid opening. As an extension of our studies to clarify the physiological role of EGF, we have identified the presence of an EGF-like substance in mouse tears and obtained evidence for its physiological role in corneal wound healing. These results raise the possible use of EGF in treating corneal wounds involving EGF deficiency in tears. In addition, by combined use of sialoadenectomy of nude mouse and anti-EGF treatment we have developed an experimental animal system to assess the role of EGF in the implantation and growth of human and animal tumors. Using this system, we have obtained the evidence indicating that EGF is necessary for the growth of mammary tumors, whereas this factor is not involved in promoting the growth of other tumors such as SV-40 transformed 3T3 cells.

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NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 57503-15 LMCB

PERIOD COVERED

October 1, 1988 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Lysosomal Transport and Storage Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I. Frank Tietze

Research Chemist

LMCB:NIDDK

COOPERATING UNITS (if any)

William A. Gahl, Research Chemist
 Section on Human and Developmental Genetics. NICHD

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

1.0

PROFESSIONAL:

1.0

OTHER:

-

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

The thyroid gland contains specialized cells capable of synthesizing the iodine-containing protein thyroglobulin, the complete hydrolysis of which in the thyroid lysosomes gives rise to its constituent amino acids, among which are moniodotyrosine (MIT), diiiodotyrosine (DIT), and the hormones triiodothyronine (T3) and thyroxine (T4). T3 and T4 leave the thyroid cell and are transported via the circulation to distant target cells. MIT and DIT also leave the lysosome but remain in the cytoplasm, where their iodine is removed for eventual reincorporation into newly synthesized thyroglobulin. We have examined the question whether a specialized carrier system is needed for transport of the iodoamino acids across the lysosomal membrane by measuring the movement of MIT in and out of purified thyroid lysosomes. The results of this study have indicated that MIT crosses the lysosomal membrane by a carrier-mediated process and explain how this product of thyroid catabolism is transported to the cytosol for iodine salvage and reutilization.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 57504-01 LMCB

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of HIV by AAV

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.: Barrie J. Carter Chief, LMCB LMCB:NIDDK

Other: Irving L. Miller Biologist LMCB:NIDDK
Nor Chejanovsky Visiting Associate LMCB:NIDDK

COOPERATING UNITS (if any)

-

LAB/BRANCH

Laboratory of Molecular and Cellular Biology

SECTION

INSTITUTE AND LOCATION

NIDDK:NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.9

PROFESSIONAL

0.9

OTHER

-

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The etiologic agent of AIDS is the human immunodeficiency virus (HIV) which differs from most other human viral diseases in exhibiting a very prolonged latent period, but ultimately being lethal due to a profound effect on the immune system. Several trans-acting HIV genes appear to be crucial to HIV growth and infection. Therefore we are studying the feasibility of a novel anti-viral therapy for HIV based on interference by another viral with the trans-acting regulation of HIV. The overall goal of this proposal is to analyze interactions between trans-acting regulatory genes of HIV and of a human parvovirus, adeno-associated virus, AAV. We are analyzing the AAV rep gene and its interaction with the HIV tat gene. Current work suggests that developing standard types of anti-viral therapy such as vaccines or nucleotide-analog drugs for HIV is difficult and other alternate possibilities for therapy must be investigated. One approach is to intervene in the trans-regulation system of HIV especially that mediated by the HIV tat gene. Thus a possible anti-viral therapy for HIV is to inhibit the production or the action of tat. A novel way to attempt this is to employ a trans-acting gene from another human virus. One such candidate is the rep gene of the human parvovirus adeno-associated virus (AAV). AAV does not cause any human disease and grows only in cells also infected with adenovirus or herpes viruses. AAV inhibits growth of the helper virus and may play an important role in limiting certain human viral infections. Also AAV can alter important regulatory controls in virus infected cells or in tumor cells. Rep is a novel type of trans-acting regulatory gene which exhibits negative, translational regulation of many genes in several cell types. We are analyzing the AAV rep gene and its interaction with the HIV tat gene. We are testing rep as a potential inhibitor of HIV infection or growth by interfering with trans-acting HIV genes.

ANNUAL REPORT OF THE LABORATORY OF ANALYTICAL CHEMISTRY

NATIONAL INSTITUTE OF DIABETES, DIGESTIVE AND KIDNEY DISEASES

SECTION ON INSTRUMENTATION

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to NIDDK scientists of the Laboratory of Chemistry, Laboratory of Bioorganic Chemistry, other NIH laboratories and to a limited extent to personnel of other government agencies. Instrumental analyses include: GC/MS spectrometry, HPLC/MS spectrometry, infrared spectrometry, nuclear magnetic resonance spectrometry, and element detection using a microwave plasma detection system. Assistance in interpretation of spectra is rendered on request. (D.F. Johnson, H.J.C. Yeh, N. Whittaker, W. White).

APPLICATIONS OF NMR IN BIOCHEMICAL AND BIOLOGICAL SYSTEMS

Colchicine, the major alkaloid of the meadow saffron *Colchicum autumnale*, inhibits microtubule assembly both in vitro and in vivo by binding with high affinity to tubulin, a major protein subunit of microtubules. In a continuation of applying nuclear magnetic resonance for elucidating molecular structures and for studying the drug-protein interaction, we have taken NMR conformational study of a number of colchicinoids and made circular dichroism measurements of natural (-)-(7S)-colchicine, unnatural (+)-(7R)-colchicine, (\pm)-colchicine and deacetamidocolchicine in the presence and absence of bovine brain tubulin. Results of the optical studies together with the NMR conformational analysis of these molecules show that colchicinoids can undergo conformational isomerization by flipping ring B and the phenyl-tropolone moiety between "aS" and "aR" configurations, and only bind to tubulin when the molecule is in an "aS" configuration. The unnatural (+)-(7R)-colchicine which has the phenyl-tropolone moiety in an "aR" configuration does not bind to tubulin. (H. Yeh, A. Brossi, M. Chrzanowska, J. Wolff, E. Hamel, C. M. Lin, F. Quinn, M. Suffness and J. Silverton).

SECTION ON STEROID HORMONES

NATURE OF STEROID-RECEPTOR INTERACTIONS

The objective of this project is to define the initial, intracellular events of steroid hormone action. These events include steroid binding to the intracellular receptor molecule, "activation" of the receptor-steroid complex to a DNA-binding and nuclear-binding species, and binding of the activated complex to those nuclear acceptor sites involved in the regulation of transcription of specific genes. One approach that we have used to examine these steps is to compare the properties of covalent dexamethasone 21-mesylate (Dex-Mes) labeled, and non-covalent dexamethasone (Dex) bound, receptors. Dex-Mes can affinity label 90% of the whole cell receptors and

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act essentially as a pure antiglucocorticoid in inhibiting Dex induction of mRNA transcripts. While the covalent complexes may bind to the biologically active sites, Dex-Mes inhibition of Dex induction was found not to occur by a competitive mechanism. In the rat hepatoma cells HTC and Fu5-5, the covalently labeled receptors were again responsible for the antagonist activity but the amount of antagonist activity in the two cell lines was different. Furthermore, the sensitivity of each cell line toward glucocorticoid induction of tyrosine aminotransferase (TAT) was different. This unequal induction of TAT in HTC and Fu5-5 cells was not due to variations in the glucocorticoid receptor and was not seen for the induction of a second primary glucocorticoid inducible gene (MMTV) at the level of mRNA transcripts in the same cells. Thus differential glucocorticoid regulation of primary gene transcripts can occur within the same cell at a pre-translational level. These results are not compatible with current models of steroid action. Further studies with this system should provide new insight into the mechanism of steroid hormone control of gene transcription. (S. Simons, Jr., P.A. Miller, H. Oshima, F.D. Sistare, T. Wasner).

THE DEVELOPMENT OF METHODS AND MATERIALS FOR THE STUDY OF MEDICAL PROBLEMS

The objective of this project is to make contributions to the investigation and solution of basic biological and medical problems by the application of chemical, physical and biological methods. Lethality from cancer frequently results from metastases. Of tumor cells which enter the circulation less than one percent are successful in negotiating the steps of metastasis. This vulnerability may afford opportunities for selectively inhibiting the process. The purposes of this study are to increase our knowledge of the biology and chemistry of metastasis and to study the effect of selected biologicals and chemicals on the process. Such studies will also contribute to the investigation of other biological and medical problems.

The stable phenotypes of many malignant cells suggest that there is a genetic basis for cancer. However, the population of malignant cells in a tumor is heterogeneous and the cells vary in metastatic potencies. Epidemiological studies and recent studies with oncogenes suggest that carcinogenesis is a multistep process. The cancer phenotype in metastasis does not represent the initial change in growth control leading to tumorigenicity. If one or more additional genetic events are required for the metastatic phenotype, they may provide approaches to the prevention or treatment of metastasis.

NIH 3T3 cells, non-tumorigenic murine cells, have been transfected with a pBR322 plasmid bearing the src gene. Transformed cells bearing the src gene were injected into nude mice subcut and iv to test for tumorigenic, metastatic and lung-colonizing capabilities. NIH 3T3 cells were similarly transfected with constructs of the v-abl, c-mos, and v-mos oncogenes. Three cell lines developed with the src gene were tumorigenic but not metastatic and had poor lung colonizing potency. Three lines of transformed cells developed from v-mos transfected cells have also been tested in nude mice. Of cell lines derived from NIH 3T3 cells by transfections with src and v-mos oncogenes and tested in nude mice, all are tumorigenic, not metastatic, and weak in producing lung colonies. These cell lines appear to be good candidates for further transfections to

determine whether greater metastatic and/or greater lung-colonizing capabilities can be developed in this way. Positive results might afford new routes for the prevention and treatment of metastases. Other means for interfering with metastasis at the various steps of the process are also being sought using murine tumor cells, such as the Lewis lung carcinoma and PMT fibrosarcoma cells. (C.M. Foltz, L.A. Liotta, R. Muschel).

SECTION ON BIOPHYSICAL HISTOLOGY

A RHODAMINE FOR INTRACELLULAR INJECTION

Studies on neuronal structure in isogenic snails, on the synthesis of a new rhodamine dye, and on the possible use of this dye as an intracellular tracer have been interrupted. (W. W. Stewart and N. Feder).

GENETICS OF NERVE CELL SHAPE

Studies are continuing on the genetics of *Biomphalaria glabrata*, a snail well suited to examining the genetic determinants of neuron structure and function. We have produced over a hundred highly inbred stains and have found about ten morphologic markers with a simple genetic basis. (W. W. Stewart and N. Feder).

PROFESSIONAL PRACTICES OF A GROUP OF BIOMEDICAL SCIENTISTS

Studies are continuing on the professional practices of scientists and on the accuracy of the scientific literature. A study completed several years ago was published last year after obstacles to publication had been overcome. This study showed a high frequency of professional misconduct in a non-randomly chosen group of biomedical scientists. A more recent study bearing on the accuracy of an article in molecular biology has been completed and submitted to three scientific journals, and has not yet been accepted for publication. We have also testified before two congressional committees on the subject of professional practices and the accuracy of the scientific literature. (N. Feder and W. Stewart).

SECTION ON BIOMEDICAL CHEMISTRY

NUCLEOSIDE AND NUCLEOTIDE ANALOGUES AS POTENTIAL ANTI-AIDS AGENTS

A redox prodrug form of the established anti-HIV agent, AZT or 3'-azido-3'-deoxythymidine, has been prepared. This material showed an ability to inhibit HIV in MT-4 cells at submicromolar concentration. In extracts of rat brain, this drug form could be converted into a positively charged species which released free AZT. When injected (i.v.) into rats, the positively charged form was found in brain at 1-3 $\mu\text{g/g}$ tissue. These results suggest that this form of AZT may effectively cross the blood-brain barrier in rats. (P. F. Torrence, J. Kinjo and K. Lesiak).

APPLICATION OF ORGANIC CHEMISTRY TO THE UNDERSTANDING OF THE INTERFERON-INDUCED 2-5A SYSTEM

A novel 2-5A/biotin affinity probe has been synthesized as an aid in the purification of RNase L. To expedite the chemical synthesis of 2'5'-oligoadenylate, a solid-phase synthetic approach has been devised which allows synthesis of 10-100 OD units of such oligomers as 2'5'-(pA)₁₀. To explore the possibility that carboxylate may be able to substitute for phosphate in the structure at the 5'-terminus of 2-5A, 5'-terminal uronic acid analogues of 2-5A have been prepared. An assay has been developed to permit quantitation of RNase L in human lymphocyte extracts. (P. F. Torrence, K. Lesiak, D. Alster, J. Kinjo, T. Kovacs).

SECTION ON MEDICINAL CHEMISTRY

COLCHICINE

Colchicine and its analogs are molecules, which combine molecular and atomic dissymmetry. Measuring the optical properties of (-)-, (±)-, and (+)-colchicine in the presence of stoichiometric amounts of tubulin showed that only the (-)-isomer did bind and that both, (±)- and (+)-colchicine afforded positive rotational signals. Positive rotational signals also were seen when deacetamidocolchicine was measured in the presence of tubulin. This clearly demonstrates that the molecular dissymmetry of these molecules is important for binding and that the latter requires presence of a *aS* configuration. All colchicinoids prepared from colchicine and having negative rotations possess this configuration. Release of steric hindrance by cleaving the 1-methoxy groups affords equilibrium mixtures of (*aS*,7*S*) with (*aR*,7*S*)-isomer with considerably reduced binding affinity. Colchicine, therefore, falls into the same category of compounds as phenyldihydrothebaines studied at the NIH some 40 years ago. The strategic importance of the 4 methoxy groups in colchicine for binding to tubulin is demonstrated with synthetic biphenyl models. Only biphenyls with a 2,3,4-trimethoxy substitution in one, and a 4'-methoxy group in the other ring, showed good binding affinity, considerably reduced in the 2'-methoxylated and weakened in the 3'-methoxy isomers. The most potent compound, however, was a tetramethoxybiphenyl which had in addition to the 4'-methoxy group a 2'-methyl substituent. The lead provided with the later compound is now fully explored. Selective ether cleavage of colchicine with BBr₃ afforded after methanolysis phenolic colchiceines, which are uninteresting for our purposes. A variety of ester derivatives of (-)-3-demethylthiocolchicine and (-)-2,3-didemethylcolchicine were prepared. The broad spectrum antitumor agent (-)-3-thiocolchicine can be directly obtained from (-)-3-demethylcolchicine. (A. Brossi, R. Alonso, M. Chrzanowska, Y. Itoh).

PHYSOSTIGMINE

Carbamate esters prepared from (-)-eseroline with isocyanates afforded with the octylcarbamate, the benzylcarbamate and the phenylcarbamate compounds, which were considerably more potent *in vitro* in inhibiting butyrylcholinesterases than Phy, however they were weaker against AChE. The only compound which displayed similar potency against both enzymes was

(-)-N(1)-norphysostigmine, a potential metabolite of Phy. Several of these ester analogs which are covered in a patent application are now being evaluated *in vivo*. Larger quantities of unnatural (+)-Phy which protects mice against organo-phosphates has been prepared by considerably improved procedures and is being further evaluated. Several analogs prepared in the (+)-series also afforded compounds which block the nicotinic acetylcholinreceptor by direct blockade more strongly than (+)-Phy. They also are included in a patent application. Attempts to N-demethylate the ethylcarbamate of (N)1-noreseroline O-methyl ether through quaternization with chloroformates or cyanogen bromide failed to give results, which would make a conversion into N(8) nor-analogs practically feasible. Synthesis of generines and physovenines by variations of the classical procedures have been started. (A. Brossi, Q. S. Yu).

MAMMALIAN ALKALOIDS

A by-product obtained from mammalian 3',4'-dideoxynorlaudanosoline-1-carboxylic acid at physiological pH has been identified as a quinone methine isomer of 1-benzyl-3,4-dihydro-6,7-dihydroxiisoquinoline. Its O-methylation with COMT proceeds as in the series of 1-carboxylic acids to 7-O-methylated products. It is therefore unlikely that TIQC or TIQ derived from them are precursors of mammalian reticuline and derived morphine. A large number of isoquinolines prepared in our programs over the years were tested for inhibition of MAO A and B *in vitro*. Only 3,4-dihydroisoquinolines showed activity against MAO A, and N-methyltetrahydroisoquinoline was the only compound which showed reasonable activity against MAO B. Selected compounds will be tested *in vivo*. (A. Brossi, M. D. Rozwadowska, M. Chrzanowska, L. Chrisey).

BETA-CARBOLINES

The 5-, 6- and 8-methoxy isomers of harmine have been prepared and are fully characterized. They will be examined for inhibition of MAO A and B. This also will include the anhydrobases prepared from harmine and 1-methyl-6-methoxy-beta-carboline. (A. Brossi, L. Chrisey).

OXINDOLES

Several oxindoles N-methylated and or substituted in the aromatic ring with chlorine will be submitted for broad pharmacological screening including testing for antifungal, anticonvulsant and anti-MAO activities. (A. Brossi, L. Chrisey).

ANTIVIRAL AGENTS

Hormothamnione from a blue green algae has been prepared by total synthesis and was tested *in vitro* for anti-HIV activity. Hormothamnione and several of its derivatives were devoid of any noteworthy activity in this assay. (A. Brossi, L. Alonso).

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ANTIMALARIALS

(+)-Primaquine prepared by the classical resolution method was converted into a crystalline urea by reaction with R-(+)-1-phenylethylisocyanate. An X-ray analysis of this urea allowed the conclusion that (+)-primaquine has the S-configuration and (-)-primaquine, therefore, is the R-isomer. Preparation of analytical standards used to detect and identify potential metabolites of arteether were prepared. (A. Brossi, L. Dominguez-Gerpe, Q. S. Yu).

The information presented in this report is for your information only and should not be used for any other purpose. It is the property of the Department of Defense and is to be kept confidential. If you have any questions, please contact the appropriate authority.

CONFIDENTIAL

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Service Functions and Instrumentation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	D.F. Johnson	Chief, Lab. Anal. Chem	LAC/NIDDK
OTHERS:	H.J.C. Yeh	Research Chemist	LAC/NIDDK
	N. Whittaker	Chemist	LAC/NIDDK
	W. White	Biologist	LAC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Instrumentation Section

INSTITUTE AND LOCATION

NIH, NIADDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS

4.0

PROFESSIONAL:

4.0

OTHER

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided)

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to NIDDK scientists of the Laboratory of Chemistry, Laboratory of Bioorganic Chemistry, other NIH laboratories and to a limited extent to personnel of other government agencies. Instrumental analyses include: GC/MS spectrometry, HPLC/MS spectrometry, infrared spectrometry, nuclear magnetic resonance spectrometry, and element detection using a microwave plasma detection system. Assistance in interpretation of spectra is rendered on request. Samples of microanalysis are handled through external contracts. (D.F. Johnson, H.J.C. Yeh, N. Whittaker, W. White).

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58001-15LAC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Applications of NMR in Biochemical and Biological Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	H. Yeh	Research Chemist	LAC/NIDDK
OTHERS:	D.M. Jerina	Sec. Chief	LBC/NIDDK
	A. Brossi	Sec. Chief	LAC/NIDDK
	P. Kovac	Research Chemist	LC/NIDDK

Cooperating Units: A.M. Acquaviva, E. Consiglio, S. Formisano, D. Liguoro, and A. Gallo (Center of Endocrinology and Oncology Experiments, Italy), J.L. Flippen-Anderson (LSM, NRL), P. Buchs (SAPEC, S.A., Fine Chemicals, Switzerland) X.D. Luo (Beijing Institute of Pharmaceutics,

China), W. Milhouse (DET, Walter Reed Army Institute of Research), and W. Peters (London School of Hygiene and Tropical Medicine, London).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Instrumentation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

6.5

PROFESSIONAL:

6.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The objective of this project is to develop and apply nuclear magnetic resonance for elucidating molecular structures and for studying the interactions within and between molecules in making contributions to the solution of various chemical problems.

Various nmr techniques have been employed: 1) to study the conformation and atropisomerization of colchicinoids. Results of nmr spectral analysis together with optical studies of (\pm)-colchicine and (\pm)-deacetamidocolchicine in the presence and absence of tubulin substantiate early hypothesis that colchicinoids bind to tubulin with the phenyl-tropolone moiety in the "aS" configuration; 2) to elucidate the structures of major adducts formed from the deoxyguanosine residues of DNA upon reaction in vitro with optically active bay-region epoxides of dibenz[A,J]anthracene; 3) to assign absolute stereochemistry at C-12 of arteether and epimer of deoxyarteether (antimalarial drug); 4) to characterize heterogeneity of the phosphate residues on thyroglobulin preparations; and 5) to study the hydrogen bonding between cytosine and peptide of threonine or serine.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58002-13LAC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nature of Steroid-Receptor Interactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. S.S. Simons, Jr., Chief, Steroid Hormones Section LAC/NIDDK

OTHERS: F.D. Sistare Staff Fellow LAC/NIDDK

P.M. Yen Intramural NRSA Fellow LAC/NIDDK

A. Cavanaugh Extramural NRSA/PRAT Fellow LAC/NIDDK

H. Oshima Visiting Fellow LAC/NIDDK

P. Chakraborti Visiting Associate LAC/NIDDK

COOPERATING UNITS (if any)

Gordon L. Hager (NCI)

E. Brad Thompson (Univ. of Texas, Galveston)

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Steroid Hormones

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

6.0

PROFESSIONAL:

5.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to define the initial, intracellular events of steroid hormone action. These events include steroid binding to the intracellular receptor molecule, "activation" of the receptor-steroid complex to a DNA-binding and nuclear-binding species, and binding of the activated complex to those nuclear acceptor sites involved in the regulation of transcription of specific genes. One approach that we have used to examine these steps is to compare the properties of covalent dexamethasone 21-mesylate (Dex-Mes) labeled, and non-covalent dexamethasone (Dex) bound, receptors. Dex-Mes can affinity label ~90% of the whole cell receptors and act essentially as a pure antiglucocorticoid in inhibiting Dex induction of mRNA transcripts. While the covalent complexes may bind to the biologically active sites, Dex-Mes inhibition of Dex induction was found not to occur by a competitive mechanism. In the rat hepatoma cells HTC and Fu5-5, the covalently labeled receptors were again responsible for the antagonist activity but the amount of antagonist activity in the two cell lines was different. Furthermore, the sensitivity of each cell line toward glucocorticoid induction of tyrosine aminotransferase (TAT) was different. This unequal induction of TAT in HTC and Fu5-5 cells was not due to variations in the glucocorticoid receptor and was not seen for the induction of a second primary glucocorticoid inducible gene (MMTV) at the level of mRNA transcripts in the same cells. Thus differential glucocorticoid regulation of primary gene transcripts can occur within the same cell at a pre-translational level. These results are not compatible with current models of steroid action. Further studies with this system should provide new insight into the mechanism of steroid hormone control of gene transcription.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58003-15LAC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Development of Methods and Materials for the Study of Medical Problems.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: C.M. Foltz Research Chemist LAC/NIDDK
 OTHERS: B. Baer Chemist LAC/NIDDK

COOPERATING UNITS (if any)

Lance A. Liotta and Ruth Muschel, Pathologists, Laboratory of Pathology, NCI

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Steroid Hormones

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.5

PROFESSIONAL

0.2

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The primary goal of this work is to contribute to the investigation and solution of basic medical problems by the application of chemical, physical and biological methods. This goal is being pursued by studies of the biology and molecular biology of murine tumor cells with emphasis on cancer metastasis. Areas of special interest are organic chemistry, biochemistry, cell biology, tissue culture, cancer biology, cancer chemotherapy and recombinant DNA methodology.

Studies are being conducted to determine whether specific gene products confer on certain tumor cells the properties required for the formation of viable metastases. NIH 3T3 cells have been transfected with constructs of several oncogenes. Transformed cells have been selected and their tumorigenic, lung-colonizing and metastatic potencies determined by subcutaneous and tail vein injections in nude mice. The correlation of these capabilities with the expression of the oncogene introduced is being investigated.

Additional transfections of certain cell lines, e.g., those with tumorigenic but not metastatic potency and with or without lung-colonizing potency will be performed in an attempt to endow the cells with the properties necessary for metastasis. Success in this would increase our knowledge of the genetic requirements for metastasis.

100

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58004-21LAC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Histochemistry: Principles, Methods and Applications

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Feder	Medical Officer (Research)	LAC/NIDDK
Others:	W. Stewart	Research Physicist	LAC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Section on Biophysical Histology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies have continued on the genetics of Biomphalaria glabrata.

Studies are continuing on the professional practices of biomedical scientists and on the accuracy of the scientific literature.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 198 to September 30, 198

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borers.)

Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: P. F. Torrence Research Chemist LAC, NIDDK

Others: D. Alster National Research Service Award Fellow LAC, NIDDK

K. Lesiak Visiting Scientist LAC, NIDDK

T. Kovacs Visiting Fellow (from 12/87) LAC, NIDDK

J. Kinjo Visiting Fellow LAC, NIDDK

COOPERATING UNITS (if any)

Foreign: U. Konstanz, W. Germany, (W. Pfleiderer); Domestic, U. of Maryland, Baltimore Co., (F. Castora); Yale University (Dr. Peter Lengyel).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Biomedical Chemistry Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS

4.0

PROFESSIONAL

4.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interferon-induced enzyme activities such as the oligo (2' 5') adenylate synthetase, The 67K dalton protein kinase and oligo (2' 5') A phosphodiesterase are investigated with a goal of understanding their role in the action of interferon, the induction of interferon by double-stranded RNA and, perhaps, control of cell growth and differentiation. Analogs of the mediator of interferon action, 2-5A, are synthesized in order to define the relationship between oligonucleotide structure and binding to and activation of the 2-5A dependent endonuclease with the eventual goal of designing useful chemotherapeutic agents based on this system.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Chemistry and Metabolism of Qinghaosu, a Chinese Antimalarial Drug

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	A. Brossi	Visiting Scientist	LAC, NIDDK
Others:	L. Gerpe Dominguez	Guest Researcher	LAC, NIDDK
	Q.-S. Yu	Visiting Fellow	LAC, NIDDK
	H. J. C. Yeh	Chemist	LAC, NIDDK

COOPERATING UNITS (if any)

Walter Reed Army Institute of Research, Washington, D. C., NIH (W. Milhous);
 Laboratory for the Structure of Matter, Department of the Navy, Washington, D. C.,
 (J. Flippen-Anderson).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Medicinal Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.1

PROFESSIONAL:

0.1

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type Do not exceed the space provided)

Structure of 11-epi-deoxyarteether formed from deoxyarteether and its α -isomer by treatment with acid in ethanol was established. It could be shown that this reaction proceeds via an intermediate vinyl ether. Treatment of deoxydihydroqinghaosu with toluene sulfonic acid in benzene afforded a dimer which was elucidated in its structure by an X-ray analysis. These compounds were made in connection with the WHO-program on arteether, an antimalarial prepared from the Chinese drug qinghaosu (artemisinin).

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physostigmine and Analogs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: Q.-S. Yu Visiting Fellow LAC, NIDDK

COOPERATING UNITS (if any)

Cornell University Medical School, N. Y., NIH (W. Riker); NIAID, NIH (J. R. Atack and S. I. Rapoport); University of Maryland Medical School, Baltimore, Maryland, (E. X. Albuquerque).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Medicinal Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.9

PROFESSIONAL

0.9

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Total synthesis of (-)- and unnatural (+)-physostigmine has been considerably improved by replacing the sodium in ethanol cyclization step with a reduction with LAH in THF. Gram quantities of (+)-physostigmine which blocks the nicotinic acetylcholin receptor ion channel and protects mice from poisoning with organo-phosphates were prepared. A series of novel carbamates of the (-)- and (+)-series were tested for inhibition of ACh and BCh and as antidotes against organo-phosphate poisoning. The octyl-, benzyl- and phenylcarbamates showed interesting biochemical properties and will be further evaluated. A sample of geneserine hydrochloride was prepared from (-)-physostigmine by oxidation with chloroperbenzoic acid and will be studied by X-ray analysis.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pyrrolidine Ant Toxins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: W. Gessner Visiting Associate LAC, NIDDK

R. Alonso Visiting Fellow LAC, NIDDK

COOPERATING UNITS (if any)

Allergic Diseases Section NIAID, NIH (M. Kowalski).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Medicinal Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892.

TOTAL MAN-YEARS.

PROFESSIONAL.

OTHER

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Synthesis of (±)-trans- and (±)-cis-2,5-dialkylpyrrolidines from the Lukes-Sorm dilactam was accomplished. (±)-Trans-2-butyl-5-heptylpyrrolidine and analogs present in form of optical isomers in several ant species were tested for the effect of vascular permeability and found to be quite active. An improved synthesis of the Lukes-Sorm dilactam was achieved.

Project terminated

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

8-Aminoquinoline Antimalarials

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	A. Brossi	Visiting Scientist	LAC, NIDDK
Others:	W. Gessner	Visiting Associate	LAC, NIDDK
	Q.-S. Yu	Visiting Fellow	LAC, NIDDK

COOPERATING UNITS (if any)

Naval Research Laboratory, Washington, D. C., NIH (J. L. Flippen-Anderson); Landau, CNRS, Laboratoire de Zoologie, Paris, France.

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Medicinal Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

S-Configuration of (+)-primaquine was established by X-ray analysis of an urea prepared with R-(+)-1-phenylethylisocyanate.

Project terminated

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED
October 1, 1987 to September 30, 1988TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Mammalian AlkaloidsPRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)
PI: A. Brossi Visiting Scientist LAC, NIDDKOthers: M. Chrzanowska Visiting Fellow LAC, NIDDK
M. D. Rozwadowska Visiting Associate LAC, NIDDKCOOPERATING UNITS (if any)
College of Pharmacy, University of Texas at Austin (C. W. Abell);
Naval Research Laboratory, Washington, D. C. (J. L. Flippen-Anderson);
Laboratory of Bioorganic Chemistry, NIDDK, NIH (C. Creveling).LAB/BRANCH
Laboratory of Analytical ChemistrySECTION
Medicinal Chemistry SectionINSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.	PROFESSIONAL	OTHER
2.2	2.2	

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Optically active 3',4'-dideoxynorlaudanosoline-1-carboxylic acids and a quinone methine obtained by oxydative decarboxylation methylate with COMT in vitro exclusively the 7-OH group. Oxidation of 1-benzyl-3,4-dihydro-6,7-dimethoxyisoquinoline to 1-benzoyl-3,4-dihydro-6,7-dimethoxyisoquinoline afforded a material useful for further study including a reduction to alcohols which were separated into erythro and threo isomers and their optical antipodes. Instability of the 1-benzoyl-3,4-dihydro compound towards acid forming instantly a imidazolium dimer was confirmed. From many isoquinolines tested for inhibition of MAO A and B the most potent inhibitors were the 3,4-dihydro compounds. Inhibition of MAO A in vitro was noted for N-methyl-1,2,3,4-tetrahydroisoquinoline. Selected 3,4-dihydro-isoquinolines will be submitted for pharmacological screening.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED
October 1, 1987 to September 30, 1988TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Structure-Activity Relationships of Colchinoids Based on Tubulin BindingPRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)
PI: A. Brossi Visiting Scientist LAC, NIDDK

Others:	M. Chrzanowska	Visiting Fellow	LAC, NIDDK
	A. Muzaffar	Visiting Fellow	LAC, NIDDK
	R. Alonso	Visiting Fellow	LAC, NIDDK
	Y. Itoh	Special Volunteer	LAC, NIDDK

COOPERATING UNITS (if any)
Division of Cancer Treatment, National Cancer Institute, NIH (E. Hamel); Laboratory of Analytical Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, NIH (H. Yeh); Sackler School, University of Tel Aviv, Israel, (M. Ravid).LAB/BRANCH
Laboratory of Analytical ChemistrySECTION
Medicinal Chemistry SectionINSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.	PROFESSIONAL.	OTHER
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Selective demethylation of colchicine and 3-demethylcolchicine has afforded a variety of phenolic colchicines and colchiceines which are all characterized. Conversion of 3-demethylcolchicine into the antitumor agent 3-demethylthiocolchicine has been achieved. Colchicine binds to tubulin as the (aS)-configured phenyltropone as shown by measuring optical rotations of deacetamidocolchicine, (-)-colchicine, (±)-colchicine and (+)-colchicine in the presence of tubulin. The absolute configuration of natural colchicine is (aS, 7S)-colchicine. A series of tetramethoxy substituted biphenyls incorporating essential structural features were synthesized and assayed for binding to tubulin. The most active compound found so far was 2,3,4,4'-tetramethoxy-2'-methylbiphenyl.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Antiviral Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: R. Alonso Visiting Fellow LAC, NIDDK

COOPERATING UNITS (if any)

Dept. of Antiviral Studies, Virology Division, Dept. of the Army, Fort Detrick, Md., (P. G. Canonico).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Medicinal Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.4

PROFESSIONAL:

0.4

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Hormothamnione from blue green algae was synthesized. This chromone compound and several of its derivatives did not show antiviral activity in the anti-HIV assay in vitro.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Beta-Carbolines

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: L. Chrisey Research Fellow LAC, NIDDK
W. Gessner Research Associate LAC, NIDDK

COOPERATING UNITS (if any)

School of Pharmacy, Dept. of Medicinal Chemistry, University of Texas at Austin
(C. W. Abell).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Medicinal Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

0.6

PROFESSIONAL.

0.6

OTHER.

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

All methoxy-substituted analogs of harmine including the 5-,6- and 8-methoxy-1-methyl-beta-carbolines have been prepared from the corresponding tryptamines. These compounds will be compared for their inhibition of MAO A and B in vitro, with harmine as a standard. This study will later be extended to a comparison of the corresponding optically active tetrahydro-congeners.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analogues of Nucleic Acids and Their Components as Potential Anti-AIDS Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: P. F. Torrence Research Chemist LAC, NIDDK

Others: J. Kinjo Visiting Fellow LAC, NIDDK
K. Lesiak Visiting Scientist LAC, NIDDK

COOPERATING UNITS (if any)

FOREIGN: Rega Institute, Catholic University of Leuven, Belgium, (Dr. E. De Clercq and J. Balzarini); Nagoya City University, Japan (Dr. K. Kohda).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Biomedical Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.5

PROFESSIONAL:

0.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To provide a brain-directed derivative of the anti-AIDS drug AZT, a redox prodrug form of this nucleoside was synthesized. Studies in extracts of rat brain have demonstrated that this material can be acted upon by rat brain enzymes to be converted into a positively charged form, which may be locked in the brain and then release AZT itself. Preliminary studies in vivo in rats showed that this compound is transported into the brain, where it is found as the positively charged prodrug form.

Studies on potential inhibitors of human immunodeficiency virus reverse transcriptase also have initiated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58015-01 LAC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Oxindoles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: L. Chrisey Research Fellow LAC, NIDDK

COOPERATING UNITS (if any)

School of Pharmacy, University of Mississippi, University, MS 38677, NIH (C. D. Hufford);

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Medicinal Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

0.2

PROFESSIONAL

0.2

OTHER

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Oxindoles prepared for testing for antifungal activity and anticonvulsant activity include the following compounds: isatin, 5-chloroisatin, 5-nitroisatin, 5-chloro-N-methylisatin, N-methylisatin and isatinic acid.

ANNUAL REPORT OF THE LABORATORY OF NEUROSCIENCE

NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

SECTION ON DRUG DESIGN AND SYNTHESIS

Design and Synthesis of Drugs Acting on Central and Peripheral Tissues

Opiates and Opiate Receptors. The opioid receptor endorphin system is believed to partially regulate the human perception of pain, pleasure, mood and certain immunological functions. Our studies aimed at design and chemical synthesis of new probes of this system have continued. Newly synthesized drugs and those prepared earlier have been utilized to continue multidisciplinary studies aimed at elucidation of the structure and function of this system. Positron Emission Tomography (PET) imaging of opioid receptor distribution in the conscious human brain has begun with [¹⁸F]cyclofoxy, a fluorinated narcotic antagonist originated in our laboratory. Highest accumulations were in the amygdala, caudate nucleus and thalamus. The unnatural enantiomer of this drug has been prepared by the NIH Opiate Total Synthesis and in the baboon brain does not localize in opioid receptor rich regions. These and other data strongly suggest that this material will be a valuable tool for determination of nonspecific binding and thus quantitation of receptor density in normal and clinically abnormal human subjects. In vitro binding and autoradiographic studies have shown that [³H]cyclofoxy binds to mu and kappa opioid receptors and confirmed the lack of binding of the unnatural enantiomer. A number of other unnatural opiates have been synthesized and are under study as possible antitussives, anticonvulsants and for use in the treatment of dystonia. Evidence for a functional mu-delta opioid receptor complex has been obtained by studies of the effects of mu and delta opioid receptor selective drugs on D-1 dopamine receptor stimulated cyclic AMP efflux from superfused rat neostriatal slices. An opioid receptor from NG108-15 cells was purified using an antibody generated against the receptor and the predominate binding is associated with a 59 kDa polypeptide chain. These findings are consistent with the labeling of a 58 kDa protein on the intact NG108-15 cells. Mu opioid receptors have been identified on the 7315c tumor cell line in studies which also showed that delta and kappa opioid receptors were absent on this cell line. These results indicate that this cell line will be a useful system in which to study the biochemistry of the mu opioid receptors without competing drug effects at delta and kappa receptors. We have developed an efficient procedure for the synthesis of the enantiomers of U50,488 and determined the absolute configuration of these compounds which are useful as probes of the kappa opioid receptor subtype. The distribution of enkephalin and dynorphin immunoreactivity and mu delta and kappa opioid receptors has been determined in the gray squirrel, guinea pig, rat and hamster. In these four rodent

species the opioid receptors are variably distributed in brain regions and opioid peptide immunoreactivity does not correlate well with opioid receptor distribution. Other studies have provided additional evidence of two classes of mu opioid binding sites in the rat brain.

Studies with Ligands for the Phencyclidine Receptor.

Phencyclidine binding sites have been implicated as allosteric sites within an ion channel which interact with glutamate receptors of the N-methyl-D-aspartate (NMDA) type. NMDA, an excitatory amino acid, acts to open these ion channels. Some phencyclidine (PCP)-like compounds have recently been reported to exert a protective effect against neuronal degeneration in ischemia; they supposedly act as antagonists against the depolarizing action of NMDA in animal brain. We have, therefore, designed and synthesized new compounds which control brain damage associated with anoxia or ischemia which typically follows stroke, cardiac arrest or perinatal asphyxia. These are N-(1-thienylcycloalkyl)alkenylamine compounds which act as antagonists to inhibit excitotoxic actions at major neuronal excitatory amino acid receptor sites. Other compounds which we have prepared may be useful anticonvulsants. Certain modifications of the piperidine ring (3,4-dehydro-PCP, PCA) or the cyclohexane ring [*trans*-(R)-3-methyl-PCA] result in compounds with similar anticonvulsant potency to PCP, but with a reduction in motor toxicity. Other PCP (*m*-nitro-PCP) and PCA analogs [e.g., *trans*-(S)-3-methyl-PCA] show reduced anticonvulsant and ataxia-inducing potency, but with a greater reduction in the latter measure, so that the therapeutic index (TI) is enhanced. A similar enhancement of the TI is enhanced with the homologous cycloalkylamines and tetrahydroisoquinolines. By modifying the basic PCP nucleus, it has been possible to obtain compounds with TIs for protection against MES seizures as high as 3-4. This can be compared to values of 1.6, 3.2, 6.9 and 8.1 for the commonly used anticonvulsants valproic acid, phenobarbital, phenytoin and carbamazepine. Some of the anticonvulsants which we have discovered may have useful anti-epileptic properties. We have discovered that structural modification of PCP, a dissociative anesthetic drug, results in compounds that protect laboratory animals from seizures with little neurotoxicity. The drugs were active in the MES test, which determines the ability of a drug to prevent seizure spread and predicts efficacy against partial seizures.

Studies with Metaphit, An Affinity Ligand for the Phencyclidine Receptor. Our studies with metaphit, which we designed and synthesized as an affinity ligand for phencyclidine binding sites, have continued with the goal of elucidating the mechanism of action of those sites. We have found that metaphit blocks PCP-induced alterations in cerebral glucose utilization in rat brain. The effects of PCP on regional cerebral glucose utilization was determined using quantitative autoradiography. PCP increased brain metabolism in selected areas of cortex, particularly limbic, and in basal ganglia and thalamus, whereas

the drug decreased metabolism in areas related to audition. These results are consistent with the known physiology of central PCP neurons and may help to suggest brain areas involved in PCP-mediated actions. Moreover, based on the behavioral similarities between PCP psychosis and an acute schizophrenic episode, these data may be relevant to the understanding of schizophrenia. The PCP-receptor-acylating agent, metaphit, blocked most of these PCP actions. In addition, metaphit by itself was found to diminish glucose utilization rather uniformly throughout brain. These results indicate an antagonist effect of metaphit on the PCP system and suggest a widespread action of metaphit, putatively at a PCP-related site, possibly in connection with the N-methyl-D-aspartate (NMDA) receptor. In another study we have found that metaphit irreversibly interacts with at least two distinct sites that mediate the pharmacological effects of PCP: the PCP binding sites linked to NMDA receptors and the dopamine uptake carrier complex. There is suggestive evidence that metaphit can act as a PCP antagonist at the latter site. Metaphit affects the inhibition of N-methyl-D-aspartate (NMDA)-induced ^3H -acetylcholine (ACh) release, ^3H -TCP binding and synaptosomal ^3H -dopamine (DA) uptake in the rat striatum.

Non-project Activity. Dr. Arthur E. Jacobson was reappointed as Chairman of the Drug Testing Program of the Committee on Problems of Drug Dependence for 1988-1989, and as Affiliate Professor in the Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University. Dr. Kenner C. Rice continues to serve as an elected member of the Executive Committee of the Organic Chemistry Division of the American Chemical Society.

SECTION ON NEUROBIOLOGY

Studies on the benzodiazepine/GABA receptor chloride channel complex

The benzodiazepine/GABA receptor chloride channel complex ("supramolecular complex") is an oligomeric group of proteins that contain recognition sites for many psychopharmacological agents including benzodiazepines, β -carbolines, barbiturates, and "cage" convulsants (such as picrotoxin). The proteins comprising this complex act in concert to regulate the activity of chloride channels that are controlled ("gated") gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the vertebrate central nervous system. Studies are in progress to characterize the molecular aspects of this system, its physiological functions and possible role in disease.

Molecular Aspects While the supramolecular complex is known to consist of subunits containing recognition sites for benzodiazepines and GABA, the number and arrangement of these subunits is not known. Furthermore, the presence of a distinct protein that is an integral component of the channel lumen has

also been proposed. Both biochemical and electrophysiological evidence suggests that "cage" convulsants act at sites in or near GABA-gated chloride channels. The exact locus of action of such compounds is important in determining the molecular arrangement of the supramolecular complex, as well as more precisely defining the site of action for depressants such as the barbiturates. The demonstration that the para-isothiocyano derivative of t-butylbicycloorthobenzoate (synthesized by B. deCosta and K. Rice) is a high affinity, irreversible ligand for sites labelled by cage convulsants suggests this compound should be useful in molecular studies of the supramolecular complex. Synthesis of a radioactive form of this compound is in progress. Studies with the organic anions picrate and niflumate, together with the demonstration that brief exposure to 302 nm ultraviolet light selectively destroys anion-supported radioligand binding to the supramolecular complex have provided novel means of examining specific anion binding sites that are constituents of the GABA-gated chloride channel. Significant differences in the biophysical properties of benzodiazepine receptors have been evinced in the Long-Sleep and Short-Sleep mouse lines. These differences were manifest in both the thermal stability of benzodiazepine receptors and temperature dependence of coupling between GABA and benzodiazepine receptors. Moreover, there no differences in radioligand binding to benzodiazepine receptors between these lines. These findings suggest that the differential sensitivities of these lines to depressants and convulsants that act at the supramolecular complex may be manifest through differences in the biophysical properties of this complex without alterations in the ligand recognition domains of these proteins. The molecular mechanisms responsible for these biophysical differences are currently under investigation.

Pharmacological Aspects Previous studies from this laboratory have employed benzodiazepine receptor "inverse agonists" as tools to examine the physiological functions of the supramolecular complex. Many of the C-3 substituted beta-carbolines used in these studies are esters, which limits their biological half-lives *in vivo*. 3-Ethoxy-beta-carboline (synthesized by J. Cook and M. Allen, Univ. Wisconsin) has been shown to be a high affinity ligand of benzodiazepine receptors with pharmacological properties of an inverse agonist. This compound appears to have a longer biological half-life than its ester analogs, and should be valuable in behavioral and neuroimmunological studies. Many structurally diverse compounds have been shown to act through the supramolecular complex. We previously reported that pharmacologically relevant concentrations of inhalation anesthetics such as diethyl ether, enflurane, and halothane increase $^{36}\text{Cl}^-$ flux through GABA-gated chloride channels in a picrotoxin sensitive fashion. A good correlation ($r=0.94$; $p<0.001$) has now been evinced between the potencies of a series of inhalation (volatile) and non-volatile anesthetics to inhibit the binding of [^{35}S]t-butylbicyclophosphorothionate (TBPS) to sites on GABA-gated chloride channels and their anesthetic potencies. Moreover, the

demonstration that Ro 15-4513 (a high affinity ligand of benzodiazepine receptors) significantly reduced methoxyflurane sleep time further supports the hypothesis that volatile anesthetics may act through the supramolecular complex.

Physiological role and implications in disease Previous studies have demonstrated that stress produces a rapid activation of GABA-gated chloride channels which may precede complete activation of the hypothalamic-pituitary-adrenal axis. The in vitro mimicry of these effect by benzodiazepines suggests this phenomenon may reflect a compensatory mechanism to perturbation of the environment. The ability of stress to mimic benzodiazepine action has now been demonstrated in a behavioral paradigm sensitive to these agents. Thus, both brief ambient temperature swim and injection affect performance in an elevated plus-maze in a fashion similar to that of anxiolytic benzodiazepines. The finding that stress-induced changes in GABA-gated chloride channels appear late in ontogeny (post-weaning) while in vitro mimicry of these changes by benzodiazepines is present at birth suggests that weaning and its attendant changes in diet and gut flora may be critical to this phenomenon. Previous electrophysiological studies (in collaboration with S. Gammal and E. Jones, DDB) in cerebellar Purkinje neurons of the galactosamine-treated rabbit model of fulminant hepatic failure (FHF) suggest that the benzodiazepine receptor is involved in the pathogenesis of hepatic encephalopathy (HE). Additional studies have been performed using another model of HE, FHF induced in rats using the hepatotoxin thioacetamide (TAA). These studies were performed in order to determine whether previously described phenomena were species or hepatotoxin-dependent. In the TAA model of FHF, administration of benzodiazepine receptor antagonists have now been shown to produce significant but transient improvement in motor activity as well as produce a partial normalization of the visual evoked response (VER). These findings coupled with the demonstration of low molecular weight substances which reversibly inhibit radioligand binding to benzodiazepine receptors in brain extracts from animals with HE suggest the benzodiazepine receptor complex may be a final common pathway in pathogenesis of HE. Further studies implicating the benzodiazepine receptor complex in control of immune function have shown that not only T-cell function, but Natural Killer cell activity (NK) can be inhibited by administration of benzodiazepine receptor antagonists.

Studies on glycine-gated channels Previous biochemical studies have evinced strong similarities between the glycine and GABA-gated chloride channels in the central nervous system. These findings are consistent with electrophysiological studies demonstrating similar anion selectivities and conductance states between these transmitter-gated anion channels. Mathematical modelling of anion effects on [3H]strychnine (a glycine antagonist) binding suggest the presence of two anion binding sites that regulate ion flow through glycine-gated chloride

channels. While the role of glycine as an inhibitory transmitter has been well established, recent electrophysiological studies suggest that glycine can augment the excitatory actions of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. This action is strychnine insensitive, and occurs at concentrations of glycine far lower than needed to produce a hyperpolarization of neurons. The observation that Mg²⁺ and other substances known to interact with NMDA-gated cation channels can modulate [³H]glycine binding in a strychnine-insensitive fashion suggest the regulation of these glycine binding sites and their relationship to excitatory NMDA-gated cation channels is feasible.

Studies on "peripheral" benzodiazepine receptors Previous pharmacological and electrophysiological studies have described the presence of recognition sites for benzodiazepines in extraneuronal tissues. These sites, referred to as "peripheral" benzodiazepine receptors, are physically and pharmacologically distinct from the benzodiazepine receptors that are components of the supramolecular complex. Using a radioactive form of AHN 086 (synthesized by A.H. Newman and K. Rice), these receptors have been covalently radiolabelled, solubilized, and isolated from rat kidney mitochondria. Two forms of the receptor were labelled with molecular weights of 48 and 32 Kdaltons on gel exclusion chromatography. The physical characteristics of these receptors are currently under study.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 - September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Receptors for Neurotransmitters and Drugs in Brain and Peripheral TissuesPRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)
P.I.: P. Skolnick Chief LN, NIDDK

Others: R.O. Trullas Visiting Fellow LN, NIDDK J.C. Marvizon Guest Worker
 LN, NIDDK E.J. Moody LN, NIDDK Guest Worker
 T.D. McIntyre Staff Fellow LN, NIDDK A.H. Lewin LN, NIDDK Guest Worker
 A.S. Basile Staff Fellow LN, NIDDK R.H. Havunjian LN, NIDDK Guest Worker
 G.E. Evoniuk Guest Worker LN, NIDDK P.K. Arora LN, NIDDK Guest Worker
 J.M. Petitto Guest Worker LN, NIDDK A. Armario Guest Worker LN, NIDDK
 E. Fride Guest Worker LN, NIDDK R.T. McCabe Guest Worker LN, NIDDK
 E. Kempner, LPB, NIDDK; E.A. Jones, S. Gammal, DDB, NIDDK; S. Paul

COOPERATING UNITS (If any)
J. Crawley, P. Sudzak, CNB, NIMH; N. Ostrowski, D. Kress, CPB, NIMH; D. Klein
LDN, NICHD; K. Waggle, VRB, DRR; A. Hauck-Newman, WRAIR; R. Collins, Jackson
Labs; J. Cook, M. Trudell, T. Hagen, M. Allen, Univ. Wisconsin; J. Barrett, USUHSLAB/BRANCH
Laboratory of NeuroscienceSECTION
Section on NeurobiologyINSTITUTE AND LOCATION
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

10.5

PROFESSIONAL

10

OTHER

0.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

High affinity, stereospecific recognition sites (receptors) for neurotransmitters, neuromodulators, and many clinically useful drugs have been identified in both peripheral tissues and the central nervous system. The interaction of a neurotransmitter, neuromodulator or drug with a specific recognition site initiates a series of events (for example, the opening of an ion channel or activation of an enzyme) resulting in either a physiological/behavioral response (in the case of a neurotransmitter or neuromodulator) or pharmacological effect (in the case of a drug). Furthermore, the presence of recognition sites for synthetic compounds suggests that endogenous substances may also be present that mimic (or antagonize) the effects of exogenously applied substances. Studies are in progress to characterize "recognition-effector" systems, to link novel recognition sites to effector systems, to identify novel endogenous and exogenous substances that modulate these sites, and to relate these systems to both physiological and pathological processes. Systems under study include: a) the benzodiazepine/GABA receptor chloride ionophore complex; b) the glycine-gated chloride ionophore; c) "peripheral" benzodiazepine receptors (in both peripheral tissues and the central nervous system) d) glycine receptors linked to NMDA-gated cation channels and e) recognition sites for compounds that regulate voltage sensitive calcium channels.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58,502-02LNS

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the brackets.)

Design, Synthesis & Drugs Acting on Central and Peripheral Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below, the Principal Investigator, L Name, title, laboratory, and institute affiliation)

PI:

K.C. Rice

Section Chief

LN-NIDDK

Co-PI: A.E. Jacobson Research Chemist

LN-NIDDK

OTHERS:

A. Newman	Guest Worker	LN-NIDDK	M. Mattson	Biologist	LN-NIDDK
B. deCosta	Fogarty Fellow	LN-NIDDK	R.J. Weber	Staff Fellow	LN-NIDDK
C-H. Kim	Staff Fellow	LN-NIDDK			
N.A. Grayson	IRTA	LN-NIDDK			
J.A. Monn	NIH Special Vol	LN-NIDDK			

COOPERATING UNITS (if any)

R Rothman (M CN), J Holaday (WRAIR), E May, L Harris, M Aceto (Medical College of VA), A Pert (M BPB), C Pert (M BPB), V Manganiello (H IR CM), JE Blalock (U Alabama), JH Woods (U Michigan), A Schoffeleer (Free U, Holland), S Larson (CC), RM Cohen (M LCM)

LAB/BRANCH

Laboratory of Neuroscience

SECTION

Drug Design and Synthesis

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

5.0

PROFESSIONAL

5.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recent recognition that the effects of diverse classes of pharmacological agents are mediated by discrete cell receptors, which normally function in concert with their endogenous ligands, has presented unique opportunities for dramatic advances in the understanding of many central and peripheral regulatory systems in animals and humans. Optimal exploitation of such opportunities requires design and synthesis of highly selective drugs as probes for study of these systems and a collaborative, multidisciplinary approach in such studies. Elucidation of the exact molecular structure and mechanism of action of these endogenous ligand-receptor systems, and the molecular mechanism of action of endogenous ligand-mimetic drugs and their antagonists will provide new opportunities for therapeutic intervention in many clinical situations and for the design of superior drugs, particularly for disorders which are now little-understood. Studies in progress are currently aimed at identification, purification, and elucidation of the structure and function of opiate, benzodiazepine and phencyclidine receptor subpopulations in the overall modulation of the CNS. These studies require synthesis of new receptor ligands for several lines of investigation utilizing: (1) irreversible ligands specific for receptor subpopulations; (2) high specific activity radiolabeled ligands for autoradiographic visualization of receptors; (3) conformationally restricted analogs of potent receptor ligands as topological probes; (4) position emission transaxial tomographic visualization of receptor patterns in living brains. Studies designed to identify a clinically useful antagonist of PCP are underway. Synthesis of previously inaccessible unnatural opiate enantiomers using the recently developed NIH Opiate Total Synthesis is also in progress with the goals of identification of new pharmacological agents and effects of opiates mediated through nonclassical opiate receptors.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders)

Design, Synthesis, and Evaluation of Medicinal Agents and Research Tools

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	A. E. Jacobson	Research Chemist	LN-NIDDK
Co-PI:	K. C. Rice	Section Chief	LN-NIDDK
Others:	A. Thurkauf	Guest Worker	LN-NIDDK
	B. DeCosta	Fogarty Fellow	LN-NIDDK
	P. Hillery	Guest Worker	FDA
	M. V. Mattson	Biologist	LN-NIDDK

COOPERATING UNITS (If any) Univ. of Michigan Med. School (J. H. Woods et al.); The Medical College of Virginia (M. D. Aceto et al.); Univ. of Chicago Med. School (W. Woolverton); Univ. of Colorado (B. J. Hoffer et al.); Naval Research Laboratory; The Committee on Problems of Drug Dependence.

LAB/BRANCH

Laboratory of Neuroscience

SECTION

Drug Design and Synthesis

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

3.0

PROFESSIONAL

2.0

OTHER:

1

CHECK APPROPRIATE BOXES)

 (a) Human subjects (b) Human tissues (c) Neither (a1) Minors (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The design, synthesis, and evaluation of ligands which interact specifically with particular CNS receptors are essential for the elucidation of the function and mechanism of action of these receptors. Phencyclidine binding sites have been implicated as allosteric sites within an ion channel which interact with glutamate receptors of the N-methyl-D-aspartate (NMDA) type. NMDA, an excitatory amino acid, acts to open these ion channels. Some phencyclidine (PCP)-like compounds have recently been reported to exert a protective effect against neuronal degeneration in ischemia; they supposedly act as antagonists against the depolarizing action of NMDA in animal brain. Studies are in progress towards the design and synthesis of new phencyclidine-like compounds which might exert protection against neuronal degeneration, and others for their anticonvulsant (anti-epileptic) effects. One of our affinity ligands for the PCP site, metaphit, has been used to study the function of the PCP site and its interaction with the excitatory amino acids. The design and synthesis of new affinity ligands for the phencyclidine receptor is in progress. Also, the affinity of various opioids for their receptors, their pharmacological study in a number of assays, as well as the pharmacological evaluation of compounds from the stimulant and depressant classes of CNS agents, has been studied under the auspices of the Committee on Problems of Drug Dependence with the goal of identifying new ligands which interact with specific receptors as agonists or antagonists, with fewer undesirable side-effects in vivo. Data from these studies have been utilized by the Expert Committee of the World Health Organization concerned with the evaluation of scientific data used for drug scheduling under the Psychotropic Substances Convention.

ANNUAL REPORT OF THE MOLECULAR PATHOPHYSIOLOGY BRANCH
National Institute of Diabetes and Digestive and Kidney Diseases

The general goals of the Branch are to investigate normal and abnormal cell function at the molecular level with emphasis on transmembrane signalling by hormones, neurotransmitters, growth factors, and other first messengers acting at the cell surface. Approaches used range from molecular biologic techniques to clinical investigation in an effort to define the pathogenesis of diseases characterized by abnormal signal transduction.

Guanine nucleotide binding proteins (G-proteins) as receptor-effector couplers

A family of G-proteins functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for polypeptide hormones, monoamine neurotransmitters, photons of light, chemical odorants, chemotactic factors, and certain growth factors. Effector functions regulated by G-proteins include cAMP formation, cGMP degradation, phosphoinositide breakdown, and several types of ion channel. Major areas of interest concerning G-proteins include: 1) definition of the diversity within this gene family; tissue and subcellular distribution; regulation of gene expression. 2) definition of domains on individual G-protein subunits involved in association of the subunits, attachment to cell membranes, interaction with receptor and effector domains, and possible interactions with other regulatory proteins. 3) definition of the degree and mechanism of specificity for individual G-proteins in coupling to both receptors and effectors. 4) definition of quantitative and qualitative alterations in G-proteins that result in altered signal transduction. Significant recent progress has been made in each of these areas:

1) G-protein diversity - cDNA cloning has defined 8 distinct types of G-protein alpha subunit to date. In several instances, the protein corresponding to a predicted cDNA product had not been defined. Using a variety of techniques including in vitro translation of cloned cDNA-derived mRNA, 2-D gel electrophoresis, transient expression of cDNAs, and studies with antisera raised against synthetic peptides, we have succeeded in identifying the products of cloned cDNAs, and in identifying their tissue and cellular distribution. We have succeeded in purifying several forms of G-protein from brain, correlating these with cloned cDNA products, and identifying a novel form of one of these proteins.

We have also cloned, sequenced and characterized the human gene for a G-protein (Gi2) alpha subunit. This has revealed the evolutionary relationship between this gene family and more distantly related GTP-binding proteins such as the ras oncogenes. Characterization of this gene will also provide insights into fundamental aspects of regulation of expression.

2) G-protein domains - Using antibodies raised against synthetic peptides that correspond to discrete regions of G-protein alpha and beta subunits, we have been able to define domains critical for

receptor interaction and membrane attachment. This approach is now being extended to definition of subunit association and effector interaction domains.

3) Specificity of receptor-effector coupling by G-proteins - Our earlier studies involved reconstitution of purified G-proteins, and receptors such as rhodopsin in artificial phospholipid vesicles to define the degree of specificity in receptor-G-protein coupling. This approach has been complemented by current studies involving stable cotransfection of G-protein alpha subunit cDNAs and cDNAs encoding distinct subtypes of muscarinic cholinergic receptor. Another approach involves the use of affinity-purified peptide specific antibodies capable of disrupting receptor-G-protein interaction. Since these antibodies can distinguish between closely related G-proteins, this approach allows identification of which receptors are coupled to a given G-protein. We have also succeeded in using peptide antibodies to immunoprecipitate an activated G-protein-effector complex (Gs-adenylyl cyclase). Extension of this approach to other G-proteins for which antibodies are available will allow identification of the corresponding effectors.

4) Altered G-proteins as a cause of altered signal transduction - Having developed tools to study G-proteins at the gene, mRNA, and protein level, we are in a position to define possible changes in G-protein expression that lead to altered signal transduction. Recent examples in which changes in G-proteins may be responsible for important alterations in signal transduction include: a) increased Gi-alpha and beta subunit expression in adipocytes from hypothyroid rats (reduced beta-adrenergic responsiveness); b) deficiency of the Go-alpha subunit in pituitary prolactin-secreting tumors that are resistant to dopamine; c) increased Gi2-alpha expression in melanoma cell line subclones that show increased motility response to chemotactic factors in vitro and increased metastatic capacity in vivo.

Pseudohypoparathyroidism (PHP)

PHP is a genetic disorder in which resistance to parathyroid hormone (PTH) may be associated with somatic abnormalities collectively termed Albright's hereditary osteodystrophy (AHO). We have previously shown that subjects with this form of PHP are resistant to multiple hormones that act by stimulating cAMP formation, and that an approximate 50% reduction in activity of the G-protein (Gs) that couples receptors to stimulation of adenylyl cyclase is present in all tissues from affected subjects. Having recently cloned the human cDNA for the Gs-alpha subunit, we were able to show that subjects with PHP show reduction in steady state mRNA for the Gs-alpha subunit. Studies are in progress to define the abnormality at the gene level that may lead to reduced gene transcription.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)

Molecular biologic studies on the cause of parathyroid neoplasia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D. Chief, Molecular Pathophysiology Branch, NIDDK

Others: E. Friedman, M.D. Visiting Fellow, MPB, NIDDK

COOPERATING UNITS (if any)

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G. Aurbach, M.D. Chief, MDB, NIDDK

J. Norton, M.D. Chief, Surg. Metab. Section, Surgery Branch, NCI

LAB/BRANCH

Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided)

Primary hyperparathyroidism (HPT) is a common endocrine disorder that can cause significant morbidity involving the renal and skeletal systems. HPT may be due to benign neoplasia of a single parathyroid gland (adenoma), benign neoplasia involving multiple parathyroid glands (hyperplasia), and rarely, to malignant neoplasia of a parathyroid gland (carcinoma). The etiology of parathyroid neoplasia has not been defined, but clinical and epidemiologic studies indicate that hyperplasia is often due to an inherited defect (multiple endocrine neoplasia types 1 and 2), and that a history of head and neck irradiation is associated with a significantly higher risk of developing parathyroid neoplasia. As with other forms of neoplasia, parathyroid tumors are presumably due to inherited (germ-line mutation) and/or acquired (somatic mutation) defects in specific genes. Etiologic genetic defects could include inappropriate expression of transforming "oncogenes" and/or loss of expression of tumor "suppressor" genes. The availability of surgically resected parathyroid tumors allows us to search for tumor-specific genetic abnormalities that may be involved in development of parathyroid neoplasia. The initial phase of this work involves comparison of genomic blots of parathyroid tumor DNA and peripheral leukocyte DNA from the same patient for rearrangements or deletions. Among the probes to be used are those for genes (e.g. parathyroid hormone gene) expressed at high levels in parathyroid tissue; rearrangements of such genes could lead to inappropriate expression of previously identified or novel oncogenes. Also, probes for genes such as that encoding the vitamin D receptor could detect deletions that abolish expression of a gene whose product prevents abnormal cell division.

NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

Guanine nucleotide binding proteins as receptor-effector couplers

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D. Chief, Molec. Patho. Branch, NIDDK
 Others: R. Collins, Ph.D. Research Geneticist MPB, NIDDK
 A. Carter, Ph.D. Senior Staff Fellow MPB, NIDDK
 P. Goldsmith, Ph.D. Research Biologist MPB, NIDDK
 W. Simonds, M.D. Senior Staff Fellow MPB, NIDDK
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COOPERATING UNITS (if any)

C. Milligan, Glasgow Univ., Scotland; H. Malech (NIAID); Y. Zick, R. Sagi-Eisenberg,
 (Weizman Institute, Israel); R. Cerione, Cornell Univ., T. Bonner, N. Buckley
 (NIMH); C. Unson, (Rockefeller Univ., N.Y.) and P. Backlund (NIMH).

LAB/BRANCH

Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

10.5

PROFESSIONAL:

5

OTHER:

5.5

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects
 (a1) Minors
 (a2) Interviews
 (b) Human tissues
 (c) Neither

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A family of guanine nucleotide binding proteins (G-proteins) functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for hormones, neurotransmitters, light, odorants, and certain growth factors. Effector functions regulated (positively and, in some instances, negatively) by G-proteins include cAMP formation, phosphoinositide breakdown, potassium and calcium channels, and cGMP degradation. We have used a variety of techniques to study the expression, distribution, regulation, structure and function of G-proteins. Our studies highlight the diversity within the G-protein family. We have purified novel G-proteins and using cloned cDNAs, defined their primary structure and distribution. We have demonstrated developmental and differentiation-dependent regulation of G-protein synthesis. Using peptide specific antibodies, in situ hybridization and northern analyses, and protein reconstitution techniques, we have defined the specificity of G-proteins in coupling to receptors and effectors. We have cloned and characterized the human gene for a G-protein to define the basis for regulation of expression. These studies provide the basis for understanding the role of G-proteins in normal signal transduction and for elucidating possible defects in G-protein structure or function as the basis for abnormal signal transduction.

NOTICE OF INTRAMURAL RESEARCH PROJECT

FORMERLY Z01 DK 59002-23 MPB
Z01 DK 43004-22 MD

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Studies on pseudohypoparathyroidism and related disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D. Chief, Molec. Patho. Branch, NIDDK
 Others: A. Carter, Ph.D. Senior Staff Fellow MPB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS

0.25

PROFESSIONAL:

0.25

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism" (PHP). Patients with this disorder differ from those with idiopathic hypoparathyroidism: they show characteristic constitutional features (Albright's hereditary osteodystrophy - AHO) and do not respond to exogenous parathyroid hormone (PTH). Subsequent to the original report, patients lacking the typical somatic features of AHO but resistant to endogenous and administered PTH have been described. In PHP, UcAMP (urinary cyclic AMP) does not increase normally in response to PTH administration. This indicated that there is a defective hormone receptor-adenylate cyclase complex in this disorder. We have now shown that many patients with PHP+AHO (PHP Ia) show an approximately 50% reduction in activity of Gs (the stimulatory guanine nucleotide binding protein associated with adenylate cyclase) in membranes from multiple tissues. Gs deficiency presumably accounts for resistance to multiple hormones in such patients. Patients with PHP without AHO show normal Gs activity (PHP Ib) and resistance only to PTH, and preliminary studies suggest a PTH receptor defect in such patients. Rare patients with PHP and AHO and multiple hormone resistance show normal Gs activity.

Using cloned human cDNA probes for the alpha subunit of Gs, we now find that steady state mRNA levels from fibroblasts of subjects with PHP Ia are reduced by approximately 50% compared with normals. Genomic cloning and other molecular biologic approaches are being used to define the genetic abnormality responsible for Gs deficiency in PHP Ia.

ANNUAL REPORT OF PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Overview

The Phoenix Epidemiology and Clinical Research Branch performs research in diabetes, obesity, digestive and kidney diseases and arthritis. The majority of the Branch activities concern diseases which are particularly prevalent among American Indians in the Southwest and the majority of the investigations performed concern these diseases among the Pima Indian population of the Gila River Indian Community. This population has the highest reported frequency of non-insulin dependent diabetes (NIDDM) in the world and a high frequency of obesity, gallbladder disease, and kidney disease that occurs as a complication of their diabetes. Other complications of diabetes such as retinopathy and neuropathy and peripheral vascular disease are associated with the diabetes. The longitudinal population based study of the Gila River Indian Community is a comprehensive investigation of the occurrence and determinants of these diseases and complications. Both genetic and environmental determinants of many of these conditions have been examined and detailed studies of the pathophysiology of both NIDDM and obesity are carried out in the clinical research facility.

The epidemiological and field investigations are conducted by the Diabetes and Arthritis Epidemiology Section. During the past year further investigations of the genetic determinants of diabetes have been performed and a major effort has been launched to identify the genetic determinants of diabetes using the techniques of restriction fragment length polymorphism analysis combined with genetic linkage studies to identify the chromosomal location of the gene or genes that are responsible for the strong degree of familial aggregation of NIDDM in multiple generations of this population.

Investigation of the long-term outcome of pregnancies in the population have shown that a high frequency of obesity and diabetes occur in the offspring of diabetic pregnancies. Recent studies have indicated that the bone age of these offspring is advanced thus providing further evidence of the far reaching implications of the diabetic intrauterine environment.

Both epidemiological and clinical research investigations of the pathophysiological mechanisms that precede the development of non-insulin dependent diabetes have been continued. Insulin resistance as measured by the euglycemic hyperinsulinemic clamp technique has been shown to be predictive of the development of impaired glucose tolerance. Impairment of glucose tolerance is also associated with an increase in weight and increasing fasting and post glucose load hyperinsulinemia. These findings indicate that the development of impaired glucose tolerance is almost certainly the result of a disorder of insulin action and that the insulin responses that accompany the changes in insulin resistance are those which would be predicted from the relationship between insulin resistance and glucose tolerance in normoglycemic individuals. These results imply that the reduced beta cell function seen in NIDDM, and previously generally believed to be the cause of the diabetes, is only manifest in the face of severe insulin resistance and may even be secondary to the mild degrees of hyperglycemia that result from the primary abnormality of insulin action.



Studies of the natural history of impaired glucose tolerance in the Pima population at large have shown that persons with impaired glucose tolerance are at high risk of developing NIDDM and that among those with impaired glucose tolerance those with the highest glucose levels and highest fasting insulin levels, but lower post-load insulin responses are those most likely to worsen to diabetes. These findings also indicate that resistance to insulin action is the primary defect which leads to non-insulin dependent diabetes and that the failure of insulin secretion occurs only in the face of pronounced insulin resistance.

Studies of insulin resistance among non-diabetic Pima Indians have shown that the distribution of insulin resistance as measured by the euglycemic hyperinsulinemic clamp is not unimodal, but shows evidence of trimodality. The trimodal frequency distribution has been shown to be consistent with the hypothesis that insulin resistance may be inherited as a result of the action of a single gene which exhibits co-dominance. Further studies have indicated that the biochemical lesion associated with insulin resistance is a post-receptor binding defect which influences glycogen storage and which is associated with abnormalities in the enzymes which are responsible for glycogen synthesis. Recent studies have indicated that insulin resistant subjects have reduced basal glycogen synthase phosphatase activity.

Several investigations have focused on vascular complications of diabetes. Proliferative retinopathy has been shown to occur in high frequency among Pima Indians with diabetes of fifteen or more years duration and specific risk factors such as hypertension, nephropathy, neuropathy, and treatment with insulin are associated with its development. Diabetic nephropathy is a frequent complication of long-duration NIDDM in the Pima Indians and excessive rates of end-stage renal disease result from it. The determinants of diabetic nephropathy and the associated renal insufficiency are not well-understood, but strong evidence of genetic determination of the propensity to develop diabetic nephropathy have been identified. The frequency of diabetic nephropathy and renal insufficiency in the diabetic offspring of diabetic parents is markedly increased thus suggesting the strong likelihood that there may be specific genetic determinants of the propensity to nephropathy independent of those of diabetes itself. Other factors which have been shown to be important in the genesis of diabetic nephropathy include blood pressure, which even before the onset of diabetes has been shown to be related to the risk of developing nephropathy many years after the onset of diabetes.

Obesity has also become an important focus of the branch. Studies using the metabolic chamber and indirect calorimetry have shown that energy expenditure measured either in the resting state or over the course of 24 hours relates to the likelihood of the development or progression of obesity. Studies have shown that resting metabolic rate, 24 hour energy expenditure and the levels of activity each show evidence of familiarity. Furthermore, we have shown that low energy expenditures predict the development of excessive increases in body weight. These observations together suggest that there are important metabolic determinants of obesity which may themselves have a genetic basis.

Other Activities

The full-time professional staff of the Branch have continued to play an active role in both national and international activities and participate in the membership of editorial boards of major scientific journals. All have been invited to give lectures or seminars to national or international organizations. The staff continued to be called upon to provide advice and assistance to outside organizations including other government agencies and universities both in the United States and elsewhere.

The Branch was unfortunate in losing the services of Dr. Barbara Howard, Assistant Chief of the Clinical Diabetes and Nutrition Section (CDNS) during the current year. She has accepted a post as Director of Research with a private medical research foundation.

The specific scientific contributions of the individual sections are summarized below.

Diabetes and Arthritis Epidemiology Section

The Diabetes and Arthritis Epidemiology Section has continued its 23-year longitudinal studies of genetic and environmental risk factors for diabetes and vascular complications of diabetes in the Pima Indians, as well as continuing data collection for epidemiological studies of rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, cholelithiasis, mortality rates and causes of death. The long follow-up provided by this study is yielding increasingly valuable data on late complications of diabetes and the transmission of risk factors for diabetes and its complications from one generation to the next. Susceptibility to diabetes appears to be transmitted by a major autosomal gene, the location of which will be sought by means of linkage analysis. To this end the laboratory has implemented a procedure for EBV transformation of lymphocytes from members of informative pedigrees. Collaborative arrangements are being finalized for study of DNA from these cells for completion of the linkage analysis.

The importance of impaired glucose tolerance (IGT) as a risk factor for diabetes has been confirmed. Even when it is transient, followed by a return to normal glucose tolerance, IGT indicates an increased risk for future diabetes. Among subjects with IGT, those who worsened to NIDDM had higher fasting and lower post-load insulin concentrations than those who did not, suggesting that insulin resistance and decreased beta cell responsiveness were important causes of the deterioration to NIDDM.

Diabetes complications are being documented and their risk factors determined. Major complications of diabetes under study are nephropathy, end stage renal disease, retinopathy, peripheral vascular disease, and periodontal disease, all of which are related to the duration and severity of hyperglycemia and appear to develop at least as frequently in this population with non-insulin dependent diabetes as in people with insulin-dependent diabetes.

The adverse affects of diabetes in pregnancy, both for the mother and offspring, are being studied. It has previously been reported that offspring

of diabetic women have more diabetes and more obesity than offspring of nondiabetic and prediabetic women. Hand and wrist x-rays have been evaluated for bone age among the offspring of diabetic women and found to be advanced relative to the bone age in the offspring of nondiabetic and prediabetic women. Thus the intrauterine environment, in addition to being an important determinant of the development of diabetes and of obesity, is also important in determining skeletal development.

The incidence and prevalence of rheumatoid arthritis (RA) is determined using clinical, serologic and x-ray data. Age-specific incidence rates are appreciably higher than reported in Caucasian and Japanese populations. Rates generally increased with age, but age-specific incidence rates were stable over a 20 year period. Among subjects without RA, rheumatoid factor in the serum predicts future development of RA, indicating that it may be an early marker for the pathogenic process which begins long before clinical signs and symptoms of RA appear.

Section staff continue to be active in medical research and education beyond the projects described here. Staff collaborate extensively in research projects conducted by the Clinical Diabetes and Nutrition Section of the branch and the National Center for Health Statistics as well as lecturing at universities and contributing to state, national, and international meetings and workshops.

Biostatistics and Data Management Section

The BDMS has been engaged in data management and support activities for the research operations of the Branch as a whole. The major activity is supporting the updating, error checking, storage, and retrieval of datasets for the extensive epidemiological study, as well as assistance with many smaller datasets from the studies conducted by CDNS. This has included work on the Phoenix Clinical Information System (PCIS) which is being programmed by the Data Management Branch, DCRT, from documentation provided by the BDMS. Progress is being made toward completing this system, with much BDMS staff time during this year being spent on verification of the accuracy of data and of the data checking routines of PCIS.

Staff of the section have also been involved in analysis and organization of other complex data systems, such as that supporting the 24h energy expenditure measurements made in environmental chamber. Other major activities include support of laboratory instrument-computer interfacing, adaptation of genetics programs written by non-NIH scientists, and extensive support of personal computers.

Two new applications of personal computers have been implemented. The first involves the maintenance of a database of diabetes diagnostic data on a personal computer at the Sacaton field clinic. Listings from this database facilitate the clinic physician's review of each patient's medical record in conjunction with previous study data to determine accurately the patient's date of diagnosis of diabetes. The second involves a demographic and pedigree database maintained on the field clinic personal computer. This allows the field staff to update demographic and pedigree data interactively and rapidly,

greatly improving the speed and accuracy compared to the old system of written reports sent to Phoenix for batch processing.

Consulting on statistical methods and data management for specific scientific projects has been the other major activity of the Section, on which much of the productivity of the direct research activities of the Branch depends.

Clinical Diabetes and Nutrition Section

Research at the Clinical Diabetes and Nutrition Section is in three major areas: non-insulin dependent diabetes mellitus, obesity and energy balance, and lipoprotein metabolism.

Pathogenesis of Non-Insulin Dependent Diabetes Mellitus

The Pima Indians of the Gila River Indian Community have the highest reported prevalence and incidence rate of non-insulin dependent diabetes mellitus of any population in the world. The diabetes occurs more often among the offspring of diabetic parents than among the offspring of non-diabetic parents, even at similar degrees of obesity in the two groups of offspring. The reasons for this parental diabetes effect as a risk factor for diabetes remains unknown. The major effort of the Clinical Diabetes and Nutrition Section continues to be a longitudinal study of the offspring of these two parental types. Adult offspring of diabetic and non-diabetic mothers are admitted to the clinical research ward for detailed metabolic studies of many aspects of in vivo and in vitro carbohydrate metabolism. Based on previously collected epidemiologic data, approximately 30% of the obese offspring of diabetic mothers are expected to develop NIDDM during a five-year follow-up period, such that it will be possible to determine 1) the metabolic characteristic(s) that is (are) predictive of the development of NIDDM and 2) document the sequence of metabolic events that occur with normal glucose tolerance, develop impaired glucose tolerance, and then diabetes.

To date, approximately 300 subjects have entered this study, about 200 subjects have been studied a second time, 140 subjects have been studied three times, 90 subjects have been studied four times, 50 subjects have been studied five times, and there are now 12 subjects who have been admitted on six different occasions. Analyses of the cross-sectional data collected from this large study have led to many important observations. Most recently, our major important observations have been that insulin resistance is not only a strong familial characteristic but that the maximal insulin-stimulated glucose disposal rate in vivo has a trimodal distribution, as does the fasting plasma insulin concentrations. Trimodal distribution of insulin action appears to be consistent with a single gene, co-dominant mode of inheritance of insulin resistance in the population.

In the past year, we have also published the first report of longitudinal observations from this study. We have analyzed the sequence of events that occurs in subjects with normal glucose tolerance and develop impaired glucose tolerance. The development of impaired glucose tolerance is associated with increasing insulin resistance and increasing plasma insulin concentrations, and no change in the percent body fat but an increase in body weight such that

there is a proportionate increase in the body fat mass and body fat-free mass. The increase in the plasma insulin concentrations associated with the development of impaired glucose tolerance were as predicted by the relationship between insulin and glucose levels among subjects with normal glucose tolerance. Thus it appeared that the subjects with impaired glucose tolerance are not insulin deficient in an absolute or relative sense.

Because of the key role that insulin resistance appears to play in the development of NIDDM, some of our attention has also been focused on the mechanism of this insulin resistance. Skeletal muscle is the site of insulin-mediated glucose disposal *in vivo* and we have continued to study the previously reported relationship between *in vivo* insulin action and insulin activation of skeletal muscle glycogen synthase activity. The major regulators of the muscle glycogen synthase activity are the kinase and phosphatases that phosphorylate or dephosphorylate the enzyme. We have recently analyzed the relationship between glycogen synthase phosphatase and insulin regulation of glycogen synthase activity. It appears that insulin-resistant subjects have a reduced basal glycogen synthase phosphatase activity in proportion to their deficit in insulin regulation of muscle glycogen synthase activity. In addition, insulin-resistant subjects also had reduced muscle glycogen concentrations following insulin infusion as well as increased muscle phosphorylase activity and glucose-6-phosphate content. Future studies in this area will be to explore further the regulators of glycogen synthase activity as well as the regulators of glycogen synthase phosphatase activity. These will include studies of insulin regulation of cyclic-AMP-dependent protein kinase activity as well as inhibitor-1 activity.

We have also assessed the relationship between insulin activation of the tyrosine kinase activity of the beta-subunit of the insulin receptor and its relationship to insulin resistance among the Pima Indians. In the past year, an assay was developed to measure the tyrosine kinase activity of the isolated insulin receptor obtained from percutaneous muscle biopsy samples of the vastus lateralis muscle. This new assay will enable us to assess the effect of abnormalities of insulin binding, insulin receptor number, and the kinase activity of the receptor itself to the insulin action in insulin-resistant states associated with impaired glucose tolerance. We will also look at the relationship between the insulin stimulation of the tyrosine kinase activity of the receptor and the insulin activation of glycogen synthase activity.

In addition, we have assessed other aspects of carbohydrate metabolism in skeletal muscle, particularly the regulation of glycolysis, by glucose-1,6-bisphosphate (GP2), a new and potentially important regulator of metabolism in skeletal muscle. The muscle biopsies were obtained before and after insulin infusion in normal glucose tolerant subjects and were analyzed for various substrates, including GP2. There was a very clear and consistent insulin-stimulated increase in GP2 that appeared to be in parallel with the insulin stimulation of glycolysis. Further studies are underway to further define the role played by GP2 in the insulin-stimulated glycolytic rate in skeletal muscle in man.

Obesity and Energy Balance

The Pima Indians have an extremely high prevalence of obesity and the obesity is a major risk factor for the development of non-insulin dependent

diabetes mellitus. For these reasons, we have been investigating the mechanisms of the development of obesity in the population. We have been studying the different aspects of the energy balance equation, particularly by studying the different components of energy expenditure in man, both in the resting condition and over 24 hours as measured in our human respiratory chamber. The data have been collected cross-sectionally and follow-up studies have now been completed on a large number of subjects. To date, the results suggest that 1) the rate of resting and 24-hour energy expenditure are familial traits, independent of individual differences in body composition, age, and sex, 2) the level of physical activity is also a familial characteristic, as is the respiratory quotient or the relative proportion of fuels oxidized over 24-hour periods. These data strongly support an important genetic factor in determining a given individual's rate of metabolism and/or rate of carbohydrate and lipid utilization throughout the day. These cross-sectional data have been very important in characterizing the determinants of energy and fuel metabolism in an individual, but the most important results in terms of the pathogenesis of obesity were obtained from prospective studies of the relationship between the different aspects of energy metabolism and body weight gain.

As published in the past year, a low resting and 24-hour energy expenditure are risk factors for body weight gain in the Pima Indian population. In addition, it appears that a low rate of lipid oxidation is also a risk factor for the development of increasing percent body fat.

Thus it appears that there are familial, and likely genetic, determinants of energy expenditure and fuel utilization rates that are predictors or determinants of the subsequent body weight gain and changes in body composition. Studies are underway to determine the mechanisms of the individual differences in energy expenditure and rates of fuel utilization--for example, the relationship between energy expenditure and catecholamine and thyroid metabolism. In addition, we plan to begin studies to assess the relationship of energy expenditure, and in particular physical activity, in the free-living conditions and subsequent gain in body weight in the population using the doubly-labeled water method which we have just begun to develop in our laboratory.

Lipoprotein Metabolism

Lipoprotein composition and metabolism in the Pima Indians are being investigated to understand the control of lipoprotein metabolism and how lipoproteins are related to obesity, insulin resistance, diabetes mellitus, and cardiovascular disease. Kinetic methods were previously developed in the laboratory for simultaneous study of VLDL, IDL, and LDL metabolism, for the short-term study of VLDL triglyceride metabolism, and for the *in vitro* evaluation of the binding properties of LDL.

Studies of the relationship between lipoproteins and insulin-mediated glucose disposal indicated that there was a significant positive correlation between VLDL levels and insulin resistance and a negative correlation between HDL concentrations and insulin action. It appeared that an obese subject's hyperinsulinemia or insulin resistance was associated with an overproduction of VLDL apoB and triglyceride, whereas in diabetes, VLDL triglyceride production was stimulated through increases in plasma free-fatty acid

concentrations or glucose levels. In vitro studies using a CHO cell-line, into which a human LDL receptor had been transfected without its promoter region (in collaboration with investigators at the University of Texas), revealed that there were differences in LDL binding between various subfractions of LDL and between IDL from individuals fed diets differing in the level of fat content. It is apparent that this system will be very useful to allow the in vitro examination of the binding properties of LDL from individuals with different levels of LDL cholesterol, and an examination of the relationship between in vitro binding and in vivo metabolic parameters of lipoprotein metabolism.

The effect of a low saturated fat, high carbohydrate diet was also studied in non-diabetic subjects and weight-matched controls. VLDL in both non-diabetics and diabetics had a higher ratio of triglyceride to apoB on the low fat diet. Production rates were quite variable but in all subjects there was a decrease in the fractional clearance rate of both VLDL triglyceride and VLDL apoB and a greater proportion of VLDL was removed without conversion to LDL. LDL production was lower and the fractional clearance rate was higher on the low fat, high carbohydrate diet. These data suggested that the mechanism for the low LDL on the high carbohydrate diet may well be a result of the up-regulation of LDL receptors leading to increased removal rates from the plasma.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69000-23 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Diabetes Mellitus and Other Chronic Diseases in the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	R.G. Nelson	Staff Fellow	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK
	D. Mott	Research Chemist	CDNS, NIDDK
	W.J. Butler	Computer Systems Analyst	BDMS, NIDDK
	H.R. Baird	Mathematician	BDMS, NIDDK

COOPERATING UNITS (if any)

Biostat. and Data Management Sec., Clinical Diabetes and Nutrition Sec., PECRB, NIDDK; Indian Health Service; Ariz. State U.; State U. of New York at Buffalo; U. of Missouri, Columbia; U. of New Mexico, Albuquerque

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS

6.2

PROFESSIONAL:

4.0

OTHER:

2.2

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The purpose of this project is to identify the determinants of non-insulin-dependent diabetes, various types of arthritis, and gallbladder disease, and elucidate the natural history of the diseases and their complications. Genetic and environmental risk factors for NIDDM and vascular complications of diabetes have been studied in the Pima Indians. The residents of the study area, currently numbering approximately 5000 people, have participated in a longitudinal population study for the last 23 years, allowing observations of the natural history of diabetes mellitus and its complications. Risk factors for obesity, hypertension, and cholelithiasis are also studied, along with the relationships of these diseases to diabetes. The genetics of diabetes is studied by means of family studies and relationships of genetic markers to disease. The roles of obesity, serum insulin concentrations, impaired glucose tolerance, occupational and leisure-time physical activity and diabetes in relatives are assessed. Risk factors for the major complications of diabetes, retinopathy, nephropathy, coronary artery disease, and peripheral vascular disease are determined by longitudinal followup of diabetic subjects. Methods for ascertainment of these complications include fundus photography, measurement of urine albumin and serum creatinine concentrations, electrocardiography, and documentation of lower extremity amputations. The severity of abnormality of glucose homeostasis is assessed by measurement of plasma glucose and serum insulin concentrations during glucose tolerance tests and measurement of glycosylated hemoglobin. This study has shown diabetes to be a serious and common disease with both genetic and environmental components. The complications, especially when involving the kidney, are an important cause of increased mortality.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69001-19 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Complications and Outcome of Diabetic and Prediabetic Pregnancies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: D.J. Pettitt Assistant Chief DAES, NIDDK

Others: P.H. Bennett Chief PECRB, NIDDK
 H.R. Baird Mathematician BDMS, NIDDK
 W.C. Knowler Chief DAES, NIDDK
 R.G. Nelson Staff Fellow DAES, NIDDK
 M.F. Saad Visiting Associate DAES, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Biostatistics and Data Management Section, PECRB.
 Karolinska Institut, Stockholm, Sweden (Foreign)
 Mayo Clinic, Rochester, Minnesota

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS

1.6

PROFESSIONAL:

0.8

OTHER:

0.8

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Macrosomia, prematurity, perinatal mortality, and congenital malformations are more common in infants of diabetic mothers than in infants of nondiabetic mothers. Offspring of diabetic women are also at an increased risk of developing obesity and glucose intolerance during childhood and young adulthood. The purposes of the project are to identify diabetes and impaired glucose tolerance during pregnancy in women in the Gila River Indian Community, to determine the effects of abnormal glucose tolerance on outcome of the pregnancy, and to determine long term prognosis for the women and their offspring. The diabetes status of every woman is determined at two-yearly intervals and during the third trimester of each pregnancy. The characteristics of women who have diabetes or impaired glucose tolerance during the pregnancy are compared to those of women who are normal during the pregnancy and subsequently develop diabetes and to those of women who remain normal. At birth, cord blood has been collected for determination of glycosylated fetal hemoglobin and proinsulin. These women and their offspring, after the age of 5 years, are followed at two-yearly intervals. It has been previously reported that offspring of diabetic women have more diabetes and more obesity than offspring of nondiabetic and prediabetic women. Hand and wrist x-rays have been evaluated for bone age among the offspring of diabetic women and found to be advanced relative to the bone age in the offspring of nondiabetic and prediabetic women. The findings suggest that the intrauterine environment, in addition to being an important determinant of the development of diabetes and of obesity, is also important in determining skeletal development.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69003-15 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Muscle Capillary Basement Membrane Thickness Prior to Onset of Diabetes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: C. Bogardus Chief CDNS, NIDDK
W.C. Knowler Chief DAES, NIDDK

COOPERATING UNITS (if any)

Department of Biology, Case Western Reserve University, Cleveland, Ohio (N.B. Rushforth) and Department of Medicine, University of California, San Francisco, California (M.D. Siperstein)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, AZ, 85014

TOTAL MAN-YEARS

0.2

PROFESSIONAL:

0.1

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Several metabolic and morphologic changes have been claimed to precede the onset of diabetes, including changes in the pattern and quantity of insulin secretion and alteration in the thickness of capillary basement membranes. This study will determine if muscle capillary basement membrane thickening is a characteristic of the prediabetic state, and if so whether the thickening is present many years before the onset of diabetes, and therefore can be considered a prediabetic marker, or whether it develops pari passu with metabolic abnormalities that occur prior to the onset of diabetic hyperglycemia. Pima Indians with two diabetic parents, and with neither patient diabetic received oral and intravenous glucose tolerance tests, and a biopsy of the quadriceps muscle from which quantitative determinations of the thickness of the capillary basement membrane with increasing age in those with diabetic parents compared to those without. The results will help to determine if vascular lesions at the level of the capillary are present before hyperglycemia develops.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69006-18 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gila River Indian Community Autopsy and Mortality Study

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: W.C. Knowler Chief DAES, NIDDK
 D.J. Pettit Assistant Chief DAES, NIDDK
 M.L. Sievers Guest Researcher DAES, NIDDK

COOPERATING UNITS (if any)

Pathology Department, Phoenix Indian Medical Center, Phoenix, Arizona

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, AZ, 85014

TOTAL MAN-YEARS

0.4

PROFESSIONAL:

0.3

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The postmortem characteristics of Pima Indians of the Gila River Indian Community are investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects. Medical records are reviewed for the determination of cause of death and for the occurrence of certain serious diseases or complications of diabetes.

The purpose of the study is to relate the outcome and cause of death to events or risk factors measured in life among Pima Indian residents of the Gila River Indian Community, particularly in relation to diabetes, cardiovascular diseases and gallbladder disease. Post-mortem examinations are obtained whenever possible on members of the Gila River Indian Community to ascertain conditions present at the time of death and ascertain cause of death as precisely as possible. In addition, death certificates and all available medical records pertaining to the subjects are obtained and reviewed in a standardized way for evidence of the complications of diabetes, vascular disease, neoplasms and other conditions, which may have been recognized prior to death. The records of the occurrence of such conditions, together with conditions recognized at autopsy, are used to determine the causes of death and incidence of complications associated with diabetes and other conditions identified initially during life by the longitudinal epidemiologic studies in the population.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69009-23 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Natural History of Arthritis and Rheumatism in the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	P.H. Bennett	Chief	PECRB, NIDDK
Others:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	W.C. Knowler	Chief	DAES, NIDDK
	R. Nelson	Staff Fellow	DAES, NIDDK
	H.R. Baird	Mathematician	BDMS, NIDDK
	A. DelPuente	Visiting Fellow	NIAMS, NIDDK

COOPERATING UNITS (if any)

Biostatistics and Data Management Section, PECRB

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, AZ, 85014

TOTAL MAN-YEARS

2.9

PROFESSIONAL:

1.3

OTHER:

1.6

CHECK APPROPRIATE BOX(ES)

(a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The development and progression of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis are being determined by means of clinical, radiographic and serological examinations carried out prospectively at two-yearly intervals among adults of the Gila River Indian Community (Pima Indians) in Arizona, in conjunction with epidemiological studies of diabetes in the same community. The purpose of this investigation is to ascertain the determinants of these diseases in the population, and to identify factors which alter the natural history of progression of the disease. Host factors such as age, sex, and various gene markers including HLA and Gm, together with various potential environmental determinants, such as obesity and evidence of exposure to infectious agents, will be studied prospectively to determine their relationship to the development of these diseases. Longitudinal data have now been collected over 20 years and represent a unique data set for epidemiological studies of arthritis.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69013-07 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Diabetes, Arthritis and Other Metabolic Diseases in the Pacific Region

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

COOPERATING UNITS (if any)

WHO Collaborating Centre for the Epidemiology of Diabetes Mellitus (P. Zimmet)
(Foreign)
South Pacific Commission (R. Taylor) (Foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS

PROFESSIONAL

OTHER

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been terminated.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69014-11 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Lipoprotein Composition and Metabolism in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: B.V. Howard Associate Chief CDNS, NIDDK

Others: B. Swinburn Visiting Associate CDNS, NIDDK

G. Ruotolo Visiting Fellow CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service;

Dept of Med, Dept of Molecular Genetics, Univ of TX, SW Med School, Dallas, TX; Dept of Med, Univ of Hiroshima Med School (foreign); Dept of Med, Univ CA, San Diego, Med School, La Jolla, CA

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS

0.25

PROFESSIONAL:

0.15

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Lipoprotein composition and metabolism in Pima Indians are being investigated to understand control of lipoprotein metabolism and how lipoproteins are related to obesity, insulin resistance and cardiovascular disease. Kinetic methods have been developed for the simultaneous study of VLDL, IDL, and LDL metabolism, for the short-term study of VLDL triglyceride metabolism, and for the in vitro evaluation of the binding properties of LDL. Studies of the relationships between lipoproteins and insulin-mediated glucose disposal indicated that there is a significant positive correlation between VLDL and insulin resistance, and a significant negative correlation between HDL concentrations and insulin resistance. These correlations were stronger in men than in women and were independent of each other. When the relationships between VLDL triglyceride and VLDL apoB metabolism were examined, the data suggested that in obese subjects hyperinsulinemia or insulin resistance induces overproduction of both VLDL apoB and triglyceride, whereas in diabetes VLDL triglyceride production is stimulated through increases in plasma free fatty acids or glucose. Obesity in the Pimas had a stronger influence on HDL in women, and the changes in HDL in obese women were associated with decreases in plasma estradiol and increases in hepatic lipase activities. Our studies on HDL suggest that HDL concentrations are related to both sex hormones and also to measures of insulin resistance and that men and women may differ with respect to the relative importance of these factors.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69015-06 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Cross-sectional and longitudinal study of "prediabetes" in the Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	B.V. Howard	Associate Chief	CDNS, NIDDK
	D.M. Mott	Research Chemist	CDNS, NIDDK
	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	B. Nyomba	Visiting Associate	CDNS, NIDDK
	B. Swinburn, M. Okubo	Visiting Associates	CDNS, NIDDK
	F. Zurlo	Visiting Fellow	CDNS, NIDDK
	G. Ruotolo, D. Freymond	Visiting Fellows	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS

8.2

PROFESSIONAL:

5.4

OTHER:

2.8

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Pima Indians of Arizona have the highest prevalence and incidence rate of non-insulin dependent diabetes mellitus (NIDDM) of any population in the world. We have been longitudinally studying a subset of this population that is at the highest risk of developing the disease. The purposes of the study are to 1) determine the metabolic characteristic which is most predictive of the subsequent development of NIDDM among non-diabetics, and 2) to document the sequence of metabolic events that occur with the transition from normal to impaired glucose tolerance and subsequently to severe hyperglycemia and NIDDM. Subjects are admitted to the clinical research ward for approximately 7-10 days to undergo a variety of in vivo tests to assess insulin action and glucose metabolism. Subcutaneous adipocytes are obtained for in vitro studies of insulin and glucose metabolism as well. The results to date have shown that hyperinsulinemia and insulin resistance appear to be predictors of the development of NIDDM. The insulin resistance is not totally attributable to degree of obesity and may well have a genetic basis. The transition from normal to impaired glucose tolerance is associated with some worsening of in vivo insulin action as well as with weight gain. The insulin response to this development of insulin resistance appears to be appropriate for the degree of glycemia such that impaired glucose tolerance is not associated with any discernable abnormalities of insulin secretion.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69016-05

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Rate-limiting steps for insulin-mediated glucose uptake in man

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus

Chief, CDNS/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIH, NIDDK, Phoenix, Arizona 85016

TOTAL MAN-YEARS

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

TERMINATE

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69018-05 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Lipoprotein Metabolism in Diabetes and the Effects of Therapy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: B.V. Howard Associate Chief CDNS, NIDDK

Others: B. Swinburn Visiting Associate CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; 2nd Dept of Med, Univ of Helsinki, School of Med, Helsinki, Finland (Foreign); Dept of Med., Institute San Raffaele, Univ. of Milan, Italy (Foreign); Dept of Med., Univ. of Naples, Naples, Italy (Foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS

0.3

PROFESSIONAL:

0.2

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The increased VLDL and decreased HDL commonly associated with non-insulin dependent diabetes are of concern because of their possible role in the etiology of the greatly increased cardiovascular disease in this disorder. This study compares VLDL and LDL metabolism in non-insulin dependent diabetics and in age and weight-matched nondiabetics. Studies were conducted in diabetics before and after therapy with sulfonylureas; also diabetics were compared on high and low fat diets. The data suggest that diabetics have abnormal VLDL and that diabetes influences VLDL-TG production independent of that of apoB. LDL concentrations in diabetics are influenced by two opposing changes - increase in direct removal of VLDL, but decrease in FCR for VLDL. Improvement of glycemic control with oral hypoglycemic therapy is followed by significant falls in VLDL-TG and LDL cholesterol and reversal of abnormalities of VLDL composition, VLDL triglyceride productions, lipase activities, and HDL subfractions. Transfer of the diabetics to a high carbohydrate, low saturated fat diet is associated with decreases in LDL, no change in HDL, and no change in VLDL in most diabetics. Metabolic studies on the two diets indicate that VLDL decreases upon removal of dietary saturated fat are due to increased clearance. The larger triglyceride rich VLDL in diabetics on a low fat, high carbohydrate diet are less efficiently converted to LDL, but clearance of both VLDL apoB and VLDL triglyceride are lower. The results indicate that high carbohydrate low fat diets can result in less atherogenic lipoproteins in most subjects with non-insulin dependent diabetes.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69019-05 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Free-fatty acid metabolism and obesity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: B.V. Howard Associate Chief CDNS, NIDDK

Others: C. Bogardus Chief CDNS, NIDDK

S. Lillioja Visiting Scientist CDNS, NIDDK

B. Swinburn Visiting Associate CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS

0.0

PROFESSIONAL

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

 (a) Human subjects (b) Human tissues (c) Neither (a1) Minors (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This study was terminated in October 1987.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69020-05 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Muscle Glycogen Synthase Activity and Insulin-Mediated Glucose Disposal

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	D. Mott	Research Chemist	CDNS, NIDDK
	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	M. Okubo	Visiting Associate	CDNS, NIDDK
	D. Freymond	Visiting Fellow	CDNS, NIDDK
	Y. Kida	Visiting Fellow	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS

1.0

PROFESSIONAL:

0.7

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type Do not exceed the space provided)

To clarify the importance of the regulation of muscle glycogen synthase to the regulation of insulin-mediated glucose storage, we have made the following observations. In subjects with low insulin-mediated glucose storage rates, both the glycogen synthase activity and the glycogen synthesis rates are reduced to one quarter of the level observed in high storage rate subjects. These results suggest that alterations in the regulation of glycogen synthase activity coincide with the altered glucose storage observed in subjects with low insulin-mediated glucose disposal rates. In muscle tissue from normal glucose tolerant subjects, glycogen concentrations increase and glucose-6-phosphate concentrations decrease with increasing insulin and insulin-stimulated glucose disposal rates. In contrast, insulin infusion in diabetic subjects was characterized by reduced insulin-stimulated glucose disposal rates associated with decreases in muscle glycogen and increases in muscle glucose-6-phosphate concentrations. These observations are compatible with insulin regulation of glucose disposal in normal subjects by stimulation of metabolism beyond the glucose-6-phosphate pool and an abnormality in insulin-stimulated glucose metabolism in diabetics which also occurs beyond the glucose-6-phosphate pool. This abnormality appears to be caused by the reduced insulin stimulation of glycogen synthase and increased phosphorylase activity following insulin infusion in diabetic subjects. The abnormal regulation of glycogen metabolism appears to be at least partially caused by low glycogen synthase phosphatase activity in insulin-resistant subjects.

NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 69021-08 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Energy Expenditure in Pima Indians: Possible Cause for Obesity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	E. Ravussin	Visiting Scientist	CDNS, NIDDK
	D. Freymond	Visiting Fellow	CDNS, NIDDK
	F. Zurlo	Visiting Fellow	CDNS, NIDDK
	L. Vaughan	Guest Researcher	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service; Metabolic Unit, Department of Medicine, University of Vermont, Burlington, VT (Dr. Elliot Danforth) Clinical Research Center, Rockefeller University, New York, NY (Dr. Rudi Leibel)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS

1.55

PROFESSIONAL:

1.15

OTHER:

0.40

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Pima Indians of Arizona have one of the highest reported prevalence of obesity and diabetes mellitus in the world. Whether body weight gain and diabetes mellitus is the consequence of a thrifty gene is not yet known. Since 1984, the different components of daily energy expenditure (sleeping metabolic rate, energy cost of arousal, thermic effect of food, and the energy cost of physical activity) have been measured in both Pima Indians and Caucasians using a human respiratory chamber. The cross-sectional and longitudinal results to date have shown that: 1) the rate of resting or 24-hour energy metabolism is a familial trait independent of individual differences in body size, age, and sex, 2) the level of physical activity, as well as the fuel mix which is oxidized over 24 hours, are also familial traits. These results support an important genetic factor in the determination of an individual's metabolic rate or fuel utilization. 3) thermic effect of food is independent of the degree of obesity and a low thermic effect of food is not a predictor of weight gain, 4) carbohydrate and protein stores are closely regulated by adjustment of oxidation to intake, whereas fat is almost exclusively used or stored in response to day-to-day fluctuation in energy balance, 5) a low resting or 24-hour energy expenditure is a risk factor for body weight gain, 6) even though peripubertal children from obese parents had similar responses to overfeeding when compared to offspring from lean parents, offspring of obese parents were more receptive to overfeeding. We are continuing to use the respiratory chamber to investigate the short-term energy metabolism in response to over- and underfeeding in adults and, in conjunction with the use of doubly-labeled water, are planning to measure the energy cost of physical activity in free-living conditions. Also, we are presently investigating the effect of age and physical fitness on energy expenditure.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69023-03 PECR

PERIOD COVERED

October 1, 1986 to September 30, 1987

TITLE OF PROJECT (90 characters or less Title must fit on one line between the borders)

Skeletal Muscle Morphology as a Determinant of In Vivo "Insulin Resistance" in Man

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus Chief CDNS, NIDDK
 Others: S. Lillioja Visiting Associate CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service
 Dept. of Physical Education, University of Texas, Austin, TX

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOXES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

This study has been completed.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69024-02 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.) WHO Collaborating Center for Epidemiological and Clinical Investigations in Diabetes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: W.C. Knowler Chief DAES, NIDDK
C. Bogardus Chief CDNS, NIDDK
D.J. Pettitt Assistant Chief DAES, NIDDK

COOPERATING UNITS (if any)

World Health Organization, Non-Communicable Diseases Program, Geneva, Switzerland (Foreign), Other World Health Organization Collaborating Centers for Diabetes (Foreign), China-Japanese Friendship Hospital, Beijing, China (Foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS

1.0

PROFESSIONAL:

0.5

OTHER:

0.5

CHECK APPROPRIATE BOXES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The WHO Collaborating Center for Design, Methodology and Analysis of epidemiological and Clinical Investigations in Diabetes was designated in 1986. The purposes of the Center are to collaborate with the World Health Organization in the implementation of the WHO/IDF action program to provide advice, consultation and collaboration with other investigators in the design, methodology and analysis of epidemiology and clinical investigations relating to the etiology and pathogenesis of non-insulin dependent diabetes (NIDDM) and its complications. The center will assist in the development and application of standardized methods for epidemiological and clinical investigations, and data analysis relating to diabetes and collaborate with those interested in applying such techniques elsewhere. The Center will advise and help in the design of new studies, including onsite assistance when necessary.

The center serves as a central laboratory for the WHO Multicenter Study of Vascular Disease in Diabetes, as well as being a participating study center for this study which is examining the mortality and incidence of vascular complications of diabetes among different ethnic groups in different countries. In addition the center has initiated a collaborative study of impaired glucose tolerance in China, and is collaborating in the preparation of a survey manual for diabetes mellitus on behalf of WHO.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69025-02 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders)

Treatment of Impaired Glucose Tolerance in Malmohus County, Sweden

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DAES, NIDDK

COOPERATING UNITS (if any)

Lund University, Dalby, Sweden (foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS

0.1

PROFESSIONAL:

0.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard un-reduced type Do not exceed the space provided.)

Mortality according to glucose tolerance was studied to determine the prognosis of impaired glucose tolerance. In 1962-65, 228,833 subjects were screened for glycosuria. Of 2477 with glycosuria, 2180 were given oral glucose tolerance tests and grouped according to normal tolerance, impaired glucose tolerance, or diabetes by World Health Organization criteria. Among subjects at least 25 years old with normal tolerance, impaired glucose tolerance, or diabetes, age-sex-adjusted mortality through 1983 was 39 ± 2 , 49 ± 4 , and 71 ± 4 deaths/1000 person-years (\pm standard error) for all causes ($p < .001$ for difference in 3 groups), and 24 ± 2 , 25 ± 3 , and 40 ± 3 for vascular causes (cardiovascular, cerebrovascular, or renal disease) ($p < .001$). 206 men with abnormal tolerance by local, but not World Health Organization, criteria were randomly assigned to diet with tolbutamide, diet only, or no treatment, which was continued through 1975. Age-adjusted all-cause mortality through 1983 did not differ significantly among treatment groups (34 ± 9 , 52 ± 10 , 45 ± 19), but vascular mortality was 10 ± 5 , 31 ± 8 , and 38 ± 19 in those assigned to tolbutamide, diet only, or no treatment ($p < .05$). Thus compared with persons with normal tolerance, diabetic subjects had higher all-cause and vascular mortality, and those with impaired glucose tolerance had higher all-cause but similar vascular mortality. Treatment of abnormal glucose tolerance apparently reduced vascular but not total mortality.

NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69026-02 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders)

Regulation of Carbohydrate and Energy Metabolism in Human Muscle

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	A. Katz	Special Volunteer	CDNS, NIDDK
	B. Nyomba	Visiting Associate	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service
 Department of Physical Education, Arizona State University, Tempe, Arizona
 Karolinska Institute, Stockholm, Sweden

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
 (a1) Minors
 (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided)

Studies were performed to investigate the regulation of carbohydrate and energy metabolism (i.e., control of adenosine nucleotides) in human skeletal muscle during exercise and euglycemic hyperinsulinemia and the regulation of lactic acid production during muscle contraction. It was found that lactate production was preceded by or occurred in parallel with increases in mitochondrial nicotinamide adenine dinucleotide reduced (=NADH), suggesting, in contrast to current views, that lactate production during submaximal exercise is oxygen-dependent. Glucose 1,6-bisphosphate (GP₂), an important regulator of key enzymes of carbohydrate metabolism, has been shown to increase after isometric contraction to fatigue (~50 seconds). To study the regulation of GP₂ contents in muscle during contraction, we obtained biopsies prior to, after 20 seconds of contraction, and at fatigue. The major increase in GP₂ occurred within the first 20 seconds of exercise, with no significant change thereafter. Preliminary results suggest that the rapid increase in GP₂ is due to activation of GP₂ synthase by its substrates G-1-P and G-6-P. The lack of continuous production, while the substrates increase, during the latter part of contraction, may be due to allosteric inhibition (by inorganic phosphate) of the synthase and/or activation of GP₂-phosphatase (by Ca²⁺ or inosine monophosphate [IMP]). A series of studies were performed to determine the in vivo regulation of AMP deaminase (deaminates AMP to IMP and ammonia), the activity of the purine nucleotide cycle (responsible for reaminating IMP back to AMP (and thereby ATP [via myokinase])). Last, it has been suggested that during euglycemic hyperinsulinemia, free glucose accumulates in the muscle of insulin-



NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69027-01 PECR

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Insulin-Receptor Tyrosine Kinase in Insulin Resistance in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	D.M. Mott	Research Chemist	CDNS, NIDDK
	B.L. Nyomba	Visiting Associate	CDNS, NIDDK
	V. Ossowski	Biologist	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Clinical Diabetes and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:

0.7

PROFESSIONAL:

0.5

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither
- (a1) Minors
- (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Insulin binding to its receptor represents the earliest event in the initiation of insulin action in target tissues. Recent in vitro studies and studies with cell clones have shown that insulin receptor displays a tyrosine kinase activity that is enhanced upon insulin binding. This insulin-activated tyrosine kinase activity is correlated with the phosphorylation status of endogenous substrates, suggesting that this might be a mechanism whereby insulin transmits signals to target cells. A defect in the insulin receptor tyrosine kinase therefore might be associated with insulin resistance. Since insulin resistance may be a predictor of the development of type II diabetes, as suggested in studies in the Pima Indians, studies of the tyrosine kinase activity of the insulin receptor were undertaken in this population.

Assays were set up to quantitatively measure insulin receptor concentration and tyrosine kinase activity in small pieces of skeletal muscle obtained by needle biopsy. Receptor concentration was estimated by ¹²⁵I-insulin binding and Scatchard analysis. Tyrosine kinase activity was determined by phosphorylation of the synthetic peptide glutamine-tyrosine with ³²P-labeled ATP. Muscle biopsies were done during a euglycemic, hyperinsulinemic clamp using two different insulin doses.

The tyrosine kinase activity of the insulin receptor increased in vivo in a dose-dependent man and correlated with insulin sensitivity. Studies are currently ongoing to confirm the findings in a larger number of subjects.



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