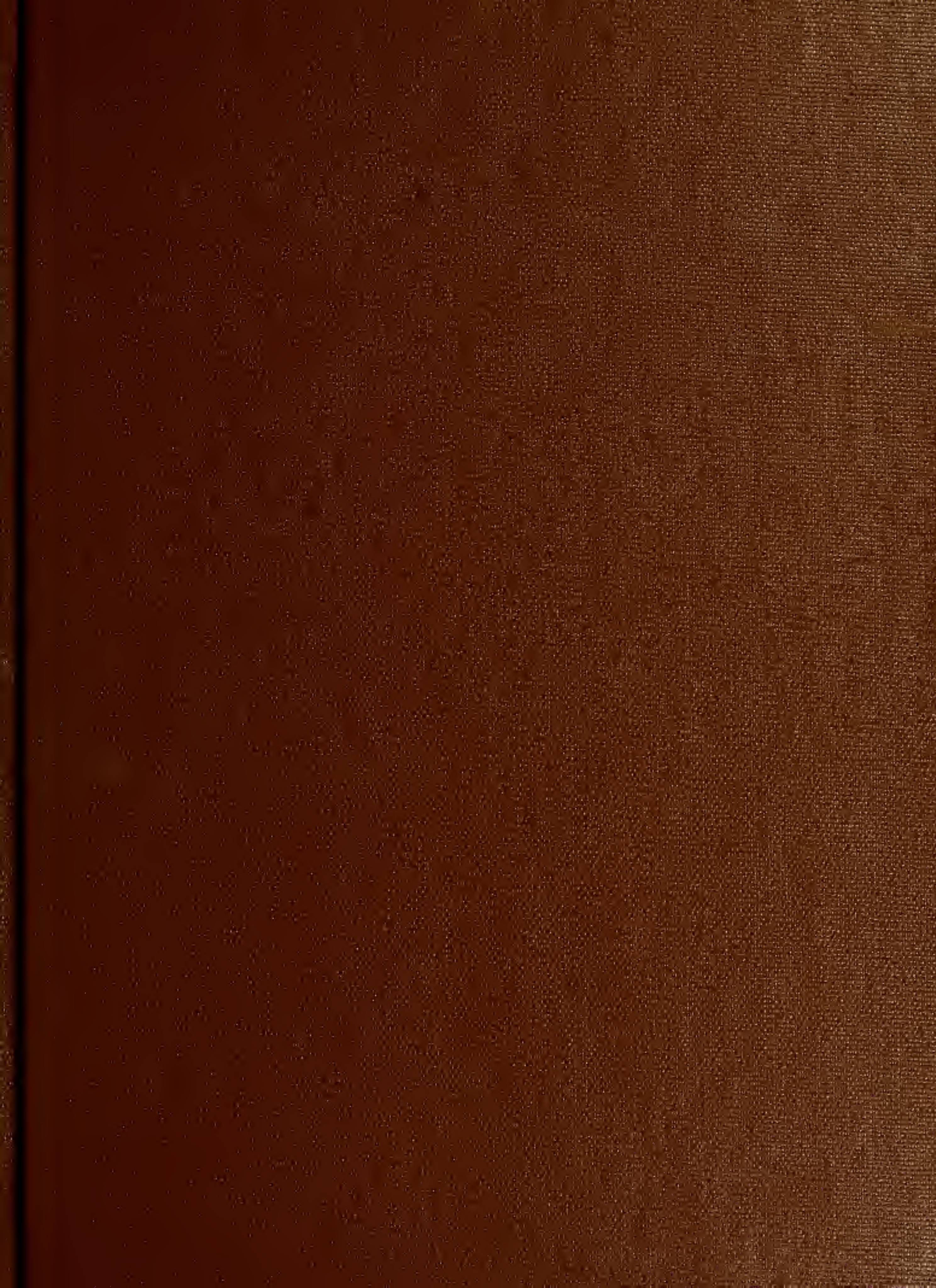

Administrative Document

Annual Report of Intramural Research Program Activities

National Institute on Alcohol Abuse and Alcoholism

Fiscal Year 1988

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration

Annual Report of Intramural Research Program Activities

National Institute on Alcohol Abuse and Alcoholism

October 1, 1987 to September 30, 1988

Summary Statements and
Individual Project Reports

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration
National Institute on Alcohol Abuse and Alcoholism
9000 Rockville Pike
Bethesda, Maryland 20892

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Annual Report of the
Division of Intramural Clinical and Biological Research
National Institute on Alcohol Abuse and Alcoholism
October 1, 1987 to September 30, 1988
Director's Overview
Boris Tabakoff, Ph.D., Director

1988 has been a solidly productive year for the NIAAA Intramural Research Program (IRP). A number of initiatives have flourished and the program has moved into several new areas of investigation. The research successes that have been achieved have been fueled by fruitful interactions between resident staff and several newly recruited staff members. The arrival of new chiefs of the Laboratory of Physiologic and Pharmacologic Studies, and of its Section of Immunology, markedly enhanced research activity and productivity, particularly with respect to alcohol's effects on the respiratory and cardiovascular systems. Novel research approaches have also been developed to study ethanol's actions on signal transduction in immune system cells. The recently recruited scientists in all three IRP laboratories have brought valuable and extensive expertise to complement that of existing staff.

Monthly Laboratory and Section Chief meetings have encouraged fruitful discussion of scientific findings among the diverse research groups in the Intramural Program. These meetings have also provided a useful forum for sharing views on administrative issues.

Prospects of improving the IRP's research facilities have also improved substantially during the past year. Renovations at the Flow building have been proceeding and safe and efficient space for conducting microbiological and molecular biological research is nearing completion. Work is also well underway to renovate the animal facility at the Flow building to meet AAALAC accreditation standards. The Flow facility will soon house a mass spectrometer for use by scientists of the Intramural Program as a whole. An automated DNA sequencer will be located there, and available to the entire IRP staff as well. The program's equipment and technological capacities have, in addition, been significantly enhanced by the recent opening of the NIH-NMR facility. The Intramural Research Program is an integral member of this multi-instrument facility, which now provides NIAAA scientists with both imaging and NMR spectroscopic capabilities.

The most promising improvement in IRP physical facilities, however, is the Child Health/ Neuroscience/Primate Facility to be

built on the NIH campus. This building, designated as Building 49, has sufficient space incorporated into its plans to permit a major consolidation of the NIAAA Intramural Program on the NIH campus. Having most of the staff in close proximity to the physical facilities and intellectual environment of NIH should decidedly enhance overall program effectiveness.

The Intramural Research staff has become committed to working closely with the extramural alcohol research community on a number of mutually beneficial programs. During the past year a consortium of extramural research programs and the IRP have planned a major research effort to unravel the role of genetic factors in alcoholism. These coordinated studies will stress electrophysiologic, biochemical, behavioral, physiologic and molecular genetic approaches in addressing one of the most exciting issues in alcohol research today. The Intramural Research Program is also selectively breeding several animal strains and lines which develop tolerance to alcohol very rapidly, as well as slowly. These experimental animals will be made available to extramural as well as intramural researchers in order to clarify the role of tolerance in the development of alcoholism.

Intramural scientists have been prominent in developing programs to provide chemical standards and materials for extramural research. Dr. Norman Salem, Jr. has chaired a collaborative effort involving NIH, ADAMHA, and the Department of Commerce to provide researchers with multiple forms of polyunsaturated fatty acids through the Fish Oil Test Materials Program. Availability of these standardized materials will permit substantially more sophisticated studies of alcohol's effects on fatty acid constituents of the cell membrane.

To promote mutual knowledge about the intramural and extramural research efforts, as well as increased interaction between intramural and extramural researchers, the Scientific Director of the IRP has presented research summaries to the NIAAA Ad Hoc Extramural Science Advisory Board at all of its regular meetings. He has also attended and made presentations at the Alcohol Research Center Directors' Meetings. The Intramural staff has been active in organizing and managing the Research Society on Alcoholism's annual meetings as well. Dr. Paula Hoffman was the Local Organizing Committee Chairperson for the 1987 meeting of the Society, and Dr. Michael Eckardt served as Chairman of the Program Committee for the Society's 1988 meeting. Both meetings were highly successful and well attended by both intramural and extramural scientists.

The Intramural Research Program Seminar Series has been expanded during the past year and there are now weekly presentations at both the Flow Building and on the NIH campus. Notable speakers during the past year included: Dr. Albert de la Chapelle from the

University of Helsinki, speaking on recent development in molecular biology of the Y-chromosome, Dr. Marion Stubbs-Spry of London's Saint George's Medical School, discussing NMR spectroscopy and histology of animal tumors, Dr. Xandra Breakefield from Harvard University, lecturing on molecular genetic studies of monoamine oxidase, Dr. Allen Spiegel from NIDDK, speaking on studies of signal transducing G proteins, and Dr. James Putney, Jr. from NIEHS, describing the relationship of inositol phosphate metabolism to cellular calcium signaling.

Intramural scientists have made major addresses at the NIH. The Scientific Director presented Clinical Center Grand Rounds on the topic of markers for alcoholism, and the Chief of the Laboratory of Clinical Studies presented a seminar on clinical investigations of alcoholism for the participants of the NIH Research Day. The Research Day activities also featured poster presentations on 10 intramural projects.

The research productivity of the IRP continues to be exemplary. Over 105 papers have appeared in peer reviewed journals and 75 other papers are in-press, awaiting publication this year. Given the overall excellence of the program, it is difficult to highlight individual projects. However, a brief discussion of a few of them can provide some sense of the breadth and imagination inherent in our Program. The Laboratory of Clinical Studies is continuing its work with families of alcoholics, assessing genetic factors predisposing some individuals to becoming alcohol dependent. The IRP has developed considerable knowledge concerning the relationships between the functioning of serotonergic systems, impulsive behavior, and alcoholism. A substantive effort has been launched to clone brain tryptophan hydroxylase and the protein which mediates serotonin uptake in platelets and neurons. Rapid progress in cloning these proteins has been achieved. Probes based on this work may be used in the near future for screening families of alcoholics for restriction fragment linked polymorphisms (RFLP). This will permit assessing a possible linkage between the genetic material coding for serotonin-related enzymes and uptake proteins and a predisposition toward impulsive behavior and alcoholism. Several other molecular genetics projects will also provide probes for future RFLP studies, and for developing more detailed knowledge of enzyme systems which are profoundly affected by alcohol. Two alcohol metabolizing enzymes have recently been cloned within the Intramural Research Program. These include the microsomal P450 isozyme responsible for alcohol metabolism, and a form of alcohol dehydrogenase that may occur in brain tissue. Researchers in the Laboratory of Metabolism and Molecular Biology have cloned various subunits of pyruvate dehydrogenate, an enzyme that may well be responsible for aberrant diol production in some alcoholics. Researchers in the Section on Immunology, in the Laboratory of Pharmacologic and Physiologic Studies, have cloned an important neuronal protein phosphatase (calcineurin), and this

work will permit more detailed analysis of calcium-dependent signal transduction processes. Considerable progress has also been made in assessing markers for alcoholism based on enzyme activities and molecular biologic techniques. Intramural researchers have focused attention on the function of adenylate-cyclase in alcoholics and have noted that the G-proteins which control adenylate cyclase activity may be aberrant in alcohol dependent individuals. Work is now proceeding to more fully determine whether such abberations are "state" or "trait" markers in the alcoholic population. These studies of markers and of molecular genetics are providing insights into the etiology of alcoholism. They also show promise as diagnostic tools for the early identification of individuals at risk for alcoholism, or of becoming problem drinkers. With such improved diagnostic tools, prevention and intervention strategies can be instituted prior to development of full-blown alcoholism.

The Intramural Research Program has made significant progress this year in understanding the underlying mechanisms by which alcohol produces both intoxication and severe organ damage. Previously unexplored neurotransmitter systems have been shown to be extremely sensitive to alcohol's central nervous system actions. Recent studies by intramural researchers have demonstrated alcohol's effects on brainstem epinephrine metabolism, and have found that inhibitors of the enzyme, catecholamine-N-methyl-transferase, can effectively reverse ethanol-induced intoxication and anesthesia. Work on alcohol's interaction with epinephrine systems holds the promise of developing novel alcohol antagonists, and a clinically useful alcohol antagonist could help prevent the many deaths that now occur from alcohol overdoses each year.

An important brain neurotransmitter system is that using glutamate as a neurotransmitter, particularly the glutaminergic system which activates the N-methyl-d-aspartate (NMDA) subclass of glutamine receptors. These receptors are linked to calcium channels and are of major importance in neural plasticity, learning and seizures. Intramural researchers using sophisticated electrophysiologic (patch-clamp) and biochemical techniques have demonstrated that the NMDA receptor-calcium channel complex, is extremely sensitive to perturbation by ethanol. These demonstrations may help explain just how alcohol affects memory processes, and they may provide further insight into how seizures are induced by alcohol-withdrawal.

Another area of intramural research (from the Laboratory of Metabolism and Molecular Biology) worthy of mention is the recent demonstration that elevated levels of tumor necrosis factor are associated with a decreased survival duration in patients suffering from alcoholic cirrhosis. Tumor necrosis factor is one of a series of lymphokines which may be related to cell death and tissue fibrosis. Demonstrating a relationship between tumor

necrosis factor and patient survival may provide a useful prognostic marker, as well as yielding valuable insights into the mechanisms responsible for liver necrosis.

Last year the Intramural Program initiated what it hopes will become an annual tradition, a State-of-the-Art Conference on a research topic of current interest and importance. The first of these Conferences was held in February of 1988 and focused on alcohol antagonists, particularly the compound RO15-4513. Through such meetings the Intramural Program will provide a forum for assessing novel research on the leading edge of progress. These conferences will also provide authoritative summaries of new research developments to the scientific and clinical community. The results of the RO15-4513 alcohol antagonist conference were published in the journal, Alcoholism: Clinical and Experimental Research.

Intramural research continues to be carefully monitored and reviewed by the Intramural Board of Scientific Counselors. Drs. Harold Kalant, Hyman Zimmerman, and Alton Meister recently completed their periods of service to the Board. Their unselfish and diligent efforts have been greatly appreciated and contributed immeasurably to creating a superb research program. During the last year, three new members were added to this Board. The current Board of Scientific Counselors includes Dr. Richard Deitrich, its Chairman, Drs. C. Robert Cloninger, Yedy Israel and Martha Vaughan, the three recently appointed members, and Drs. Jack Mendelson and William Lands, continuing members of the Board.

The quality of the Intramural Research efforts is further attested to by the invitations that members of the program continue to receive to participate in major national and international meetings, and to organize sessions and symposia within such meetings. Some recent examples of such activities include: Invited presentations of scientific findings at meetings of the Research Society on Alcoholism, the American Society for Clinical Pharmacology, Winter Conference on Brain Research, the American College of Neuropsychopharmacology, the American Psychiatric Association, the International Conference on Integration of Mitochondrial Function, the 4th International Conference on Cognitive Neuroscience, the 6th International Symposium on calcium-binding proteins in Health and Disease, the 2nd International Symposium on Medicinal Drugs and Driving Performance and the 4th Congress of the International Society for Biomedical Research on Alcoholism.

The program has been honored on several occasions during this last year by awards presented to its staff. Dr. Richard Lister received the Merck Sharp & Dohme Research Prize, presented annually to an outstanding young psychopharmacologist. Dr. Markku Linnoila received the Executive Meritorious Service Award

for his clinical and basic science research accomplishments. The Scientific Director was honored twice during the past year, once with the Research Society on Alcoholism Award for outstanding research accomplishments, and then by receiving the Jellinek Prize for his research contributions on the neurochemical effects of alcohol.

Another notable feature of the Intramural Research Program is its commitment to training young scientists. Both predoctoral and postdoctoral researchers are ably trained by the senior staff of the IRP. Young doctoral researchers from various parts of the world as well as the United States have received alcoholism research training in the Program. Currently, there are fifteen Fogarty Fellows from such diverse geographical areas as Argentina, Japan, Finland, and Zimbabwe participating in the Division's program. The program is also looking forward to being able to train additional American doctoral graduates through the Intramural Research Training Award Program. It is hoped that this program will be in place in 1989 after Congressional reauthorization action for the Institute. The IRP is also an excellent training site for pre-doctoral students and even undergraduates interested in alcoholism related research. A number of programs are utilized to provide such training, including arrangements with local universities for training doctoral students, summer fellowship programs, and minority-access-to-science training programs.

The Intramural Research Program staff has also made notable efforts to transmit results of its research program, as well as alcoholism research news from the research field at large, to lay audiences and treatment specialists. Presentations have been made at the National Council on Alcoholism meetings, the Texas Commission on Alcoholism meetings, and arrangements have been completed with the National Association of Alcoholism and Drug Abuse Counselors to provide research summaries for the Association's national journal, The Counselor. It is hoped that these efforts, together with press contacts and educational efforts within the medical training programs of universities and the NIH-FAES, will solidify a lay as well as professional understanding of alcoholism research and generate the appropriate and necessary support for researchers' efforts. The following summaries of the work of the various laboratories of the Intramural Research Program provide additional detail regarding our research endeavors and accomplishments, as the program looks forward to another year of substantive progress towards reducing the damage being caused to society by alcohol abuse and alcoholism.

Annual Report of the
Laboratory of Clinical Studies
Division of Intramural Clinical and Biological Research
National Institute on Alcohol Abuse and Alcoholism
October 1, 1987 to September 30, 1988
Markku Linnoila, M.D., Ph.D., Chief

Introduction

During fiscal year 1988, investigators in the Laboratory of Clinical Studies (LCS) continued conducting the longitudinal projects outlined in previous annual reports, and started new projects to study panic disorder in alcohol dependent patients, alcohol withdrawal, and alcohol dependence in women. Recent research efforts in LCS have more heavily concentrated on the genetics of alcoholism, on impulsivity, and on violent behavior. Special projects have been started concerning American Indians and Blacks.

The Laboratory is also investigating the effects of alcohol on the central nervous system and the liver, and complications of alcohol abuse and dependence. Moreover, it is testing novel approaches to improving the maintenance of abstinence in alcohol dependent patients.

The Laboratory of Clinical Studies administrative structure supporting its comprehensive clinical research program has been strengthened in 1988. Information relevant to improving the prevention and treatment of alcohol dependence and its complications is being gathered. Findings indicative of differences in stress responsive physiological systems between alcohol dependent patients and controls are particularly important. Furthermore, relatively specific serotonergic deficits have been found to play a role in antisocial behavior associated with alcoholism and in alcohol-induced amnestic syndrome (Korsakoff's psychosis).

I. Section of Clinical Science

a. The main objectives of the research conducted in this section are to:

1. characterize the early and prolonged components of alcohol withdrawal in alcohol-dependent patients;

2. elucidate physiological and psychological abnormalities present in patients with alcohol amnestic syndrome and alcohol-induced dementia, and to devise a pharmacological intervention to improve memory defects;

3. describe and understand the behavioral and biochemical interaction between alcoholism and panic attacks, depression and suicidal behavior;

4. compare similarities and differences between alcohol dependence and compulsive gambling;

5. explore mechanisms of social drinking-induced blood pressure elevations;

6. explore the relationship between the effects of alcohol and gender using a number of challenge studies; and

7. introduce new pharmacological interventions for the treatment of alcoholism.

b. Recent findings:

Studies involving alcohol withdrawal have focused on the assessment of both central and peripheral indices of sympathetic/stress axis response. Findings indicate that alcohol withdrawal results in a dysregulation of cortisol and catecholamine metabolism. It is reasoned on the basis of animal studies that these changes may have a pathological effect on the central nervous system and predispose to psychopathology.

Studies involving patients with the alcohol amnestic syndrome have been primarily aimed at characterizing the deficit and studying possible pharmacological interventions to enhance memory functioning. Our approach has been to treat subjects with fluvoxamine, a serotonin re-uptake inhibitor. Preliminary results suggest that certain aspects of memory improve after several weeks of fluvoxamine treatment. In a descriptive study we reported that subjects with a dual diagnosis of alcoholism and panic disorder were unable to distinguish between a number of symptoms common to both disorders. This lead us to perform a number of challenge paradigms to study the relationship between alcoholism and panic attacks. Administration of sodium lactate, a commonly used probe to provoke panic attacks in susceptible individuals, reveals that alcoholics with panic disorder are subsensitive in their response compared to non-alcoholic patients with panic disorder. This suggests that panic attacks in alcoholics may have a different biochemical etiology than that in panic patients who are not alcoholic. Secondly, a study examining the cerebrospinal fluid from alcoholics with and without panic disorder has shown that those patients with panic disorder have higher levels of endorphins. The significance of this finding requires further investigation. Other studies looking at the effects of chlorimipramine (serotonin re-uptake blocker) and

isoproterenol (beta agonist) are currently in the process of being analyzed.

The effects of alcohol on the regulation of neuroendocrine functioning in females is still in an early stage of investigation.

A study employing precursors to dopamine and serotonin was designed to reduce an alcoholic's craving for alcohol. To date, 45 subjects have entered the study and eight have remained abstinent for one year. Data analysis will be completed when the last subject finishes the study in November, 1988.

c. Future studies. New studies are designed to understand:

1. the effects of alcohol on the limbic structures (emotional centers) of the brain;

2. the role of serotonin in determining the behavior patterns of alcoholics; and

3. the effect of childhood environment on the psychopathology of adult children of alcoholics.

II. Unit of Family Studies

The family studies program evaluates subjects for genetic, physiological and biochemical studies conducted by other investigators in the Laboratory. Research currently in progress includes correlational studies comparing different subgroups of alcoholics in order to elucidate risk factors for alcoholism and other impulsive behaviors such as suicide attempts, physical violence and drug abuse. A study comparing family functioning in the families of middle-class Black alcoholics and those of non-alcoholics is in progress. The program is also in the process of phenotyping large pedigrees of alcoholic American Indians, Blacks and Caucasians in collaboration with the Section of Genetic Studies.

III. Section of Genetic Studies

The section of genetic studies is performing genetic linkage studies on alcoholism and molecular cloning studies on tryptophan hydroxylase and alcohol dehydrogenase.

By using the genetic linkage approach, this group recently mapped a possible gene for ethanol intake in the mouse (LTW-4) to mouse chromosome one.

The human linkage studies aim at detecting genetic determinants for alcoholism in families carefully characterized in collaboration with the Unit on Family Studies, and the Section on Clinical Brain Research. A large number of random polymorphic

DNA and protein probes are being applied to these pedigrees in collaboration with Dr. S. O'Brien of the National Cancer Institute. In addition, several candidate locus probes are being used to test specific hypotheses.

Human class III alcohol dehydrogenase was cloned and its sequence and expression analyzed. This enzyme is expressed in all human and mouse tissues we have examined. Studies at the enzyme level reveal the probable role for Class III alcohol dehydrogenase is in the metabolism of omega hydroxy fatty acids and long chain alcohols. A Sac I polymorphism was found. We have shown that all three class I alcohol dehydrogenase genes reside on a single fragment of genomic DNA which is less than 1 million base pairs in size. Work continues on this gene complex.

A tryptophan hydroxylase was cloned from a mouse mastocytoma cDNA library and its expression analyzed. This cDNA shows considerable sequence similarity to other aromatic amino acid hydroxylases such as phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylases cloned from other species. The mastocytoma, tryptophan hydroxylase, is highly similar or identical to the tryptophan hydroxylase of the pineal gland, but distinct from the enzyme expressed in the raphe nucleus. Work continues towards isolating the tryptophan hydroxylase of brain.

IV. Section of Clinical Brain Research

Investigators in the area of clinical brain research conduct sophisticated electrophysiological, neuropsychological, and brain imaging studies on alcoholics, individuals at risk and abstinent carefully matched controls. Variables related to man-machine interactions and relevant to driving and occupational safety while intoxicated are also being studied. Novel and potentially effective countermeasures to reduce the safety risks of alcohol consumption in driving and occupational task performances will be tested. New strategies to reverse the cognitive deficits associated with acute alcohol intoxication and with alcoholism-associated organic brain syndromes are being explored. The acute effects of alcohol on learning and memory functions are being investigated. In addition, animal studies are being conducted on the behavioral effects of alcohol, and the actions of alcohol antagonists.

V. Section of Clinical Biochemistry and Pharmacology

Because the liver is one of the major targets of alcohol-induced organ damage, researchers in clinical biochemistry and pharmacology are studying liver function and the effects of impaired liver function on the pharmacokinetics and pharmacodynamics of common medications in alcohol dependent patients. These studies are expected to provide rationales for individualized drug dosing to treat patients with varying degrees of alcohol-induced liver injury. The long-term goal is to

develop a noninvasive liver function test based on measurements of labeled carbon dioxide in breath after administration of ^{13}C-labeled drugs.

Pharmacokinetic principles and drug clearance concepts are being applied to studies of disposition of neurotransmitters and their metabolites in humans, in vivo. The goal is to define new testable hypotheses about the biochemical bases of alcohol dependence and mental disorders, and the mechanisms of action of drugs used in their treatment. These methods have for the first time been used to characterize the pharmacokinetics of norepinephrine in humans. The results of such analyses are helpful in interpreting the meaning of concentrations of norepinephrine and its metabolites in various body compartments.

VI. Section of Analytical Chemistry

Research in the Section of Analytical Chemistry concerns modification of biological functions of polyunsaturated lipids by alcohol. Both plasma membrane structure and fatty acid metabolism are being examined. Decreases in the levels of polyunsaturates in blood cells and tissues have been observed in rats after alcohol inhalation. Dietary supplementation with increased levels of these lipids helps in preventing this deficiency. Possible losses of omega-3 fatty acids from the brain after chronic alcohol exposure are also being investigated, as is the prevention of such neuronal lipid deficiencies by incorporating omega-3 fatty acids into the diet. Prostaglandins and other eicosanoids are measured in the cerebrospinal fluid and saliva of alcoholics and normal volunteers. Saliva has been shown to contain prostaglandin E2, and a study of possible uses of this observation for the facile monitoring of eicosanoid status of alcoholics is in progress. Investigations are also underway concerning the molecular species composition as well as the topology of species alterations in red cell plasma membranes. A method has been developed for the labeling of cell surface phosphatidylethanolamines (PE) by the use of covalent probes for membrane fine structure. It has been observed that human red blood cell PE is asymmetrically distributed with the more unsaturated species preferentially localized on the cytoplasmic leaflet of the plasma membrane. This assay will now be applied in clinical studies involving red blood cells of alcoholics. Further progress has been made in characterizing a novel series of leukotriene-like compounds formed in the mammalian central nervous system or in platelets from 22:6w3 fatty acids. Hydroxylated 22:6w3 fatty acids are produced in a steroselective manner by platelet 12-lipoxygenase. This fatty acid metabolite is capable of a spasmogenic action on smooth muscle preparations and, more importantly, may antagonize the contractile effect of thromboxane agonists. Treatment of smooth muscle preparations with, for example, 14-hydroxy-22:6w3 fatty acid led to the stimulation of peptidyl-leukotriene release into the perfusate. Chronic treatment with alcohol leads to a marked increase in the capacity of platelets to produce hydroxylated 22:6w3 derivatives.

These studies may lead to the elucidation of new mechanisms which regulate cardiovascular and brain function and an understanding of their disruption in alcoholism.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00238-06 LCS

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.)

CSF Neuropeptides and Prostaglandins in Alcohol Withdrawal and Brain Disease

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

PI:	M. Linnoila	Chief	LCS, NIAAA
Others:	J. Yergey	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

Laboratory of Clinical Neurogenetics, NIMH (W. Berrettini); VA Medical Center, Washington, D.C. (J. Hawley)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	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.)

Severity of withdrawal symptoms from alcohol was quantified in alcoholics admitted to the Neurology Ward of the Washington, D.C. VA Hospital. Cerebrospinal fluid (CSF) samples were repeatedly obtained early during withdrawal and after all symptoms had subsided. Concentrations of the monoamine neurotransmitter norepinephrine and its major metabolite MHPG were measured at NIH. Significant positive correlations were observed between indices of elevated norepinephrine turnover and several signs of alcohol withdrawal. We are continuing this work trying to identify causes for the noradrenergic dysregulation during alcohol withdrawal. Thus, we are measuring peptides and prostaglandins, known to participate in the regulation of the functioning of noradrenergic synapses simultaneously with norepinephrine. We are correlating the concentrations of these neuromodulators to concentrations of norepinephrine and MHPG in the CSF and to the severity of withdrawal symptoms in our patients. We have completed this phase of the study in ten patients and are aiming to increase our sample size to fifteen.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION:

Investigators:

M. Linnoila	Chief	LCS, NIAAA
J. Yergey	Senior Staff Fellow	LCS, NIAAA
W. Berrettini	Senior Staff Fellow	LCNG, NIMH
J. Hawley	Neurologist	VA Medical Center

Objectives:

Many symptoms of alcohol withdrawal are indicative of sympathic nervous system overactivity (excitability, tremor, sweating, etc.). The activity of the sympathetic nervous system is controlled by central nervous circuits, which use norepinephrine as transmitter. We are investigating central noradrenergic activity in alcoholics undergoing severe withdrawal, and correlating the degree of clinical symptoms with biochemical changes indicative of the amount of norepinephrine released from presynaptic nerve terminals. Furthermore, we are measuring peptide and prostaglandin neuromodulators known to participate in the regulation of the noradrenergic neurone systems.

Methods Employed:

Clinical symptoms of withdrawal are rated by Dr. James Hawley in the Neurology Department at the Washington, D.C. VA Hospital where the patients are treated and studied under an VA approved protocol. The instrument used to quantify severity of individual withdrawal symptoms, as well as the total score, is the extensively validated Gross rating scale. Dr. Hawley also performs two LPs on every subject, one early and the other one late during withdrawal. CSF norepinephrine and MHPG concentrations are quantified in the Laboratory of Clinical Studies, NIAAA with liquid chromatography using electrochemical detection. Prostaglandins are quantified with mass fragmentography. Neuropeptides are measured with radioimmunoassays by Dr. Berrettini.

Major Findings:

CSF norepinephrine and MHPG concentrations were significantly ($p<.01$) higher in the early than in the late CSF samples obtained from alcoholics during withdrawal. The concentrations in the early but not in the late samples were significantly ($p<.01$) higher than norepinephrine or MHPG concentrations in the CSF of an age and sex matched control group, which consisted of neurological patients. CSF norepinephrine and MHPG concentrations related highly with each other and with the severity of sweating, anxiety, tremor, heart rate and blood pressure in the withdrawing alcoholic patients.

Significance to Biomedical Research and the Program of the Institute:

These results provide the strongest available evidence of significant central noradrenergic overactivity during alcohol withdrawal in humans. Furthermore, the significant associations between the biochemical results and clinical symptoms are suggestive of the role noradrenergic overactivity plays as a possible cause of these symptoms.

Proposed Course:

We are collecting cerebrospinal fluid, urine, and blood samples in patients with alcohol-induced chronic brain disease and in a new group of patients undergoing withdrawal from alcohol to investigate possible monoaminergic deficits. Such deficits, if found, will provide rationale for treatment strategies. Furthermore, we are quantifying prostaglandins and neuropeptides known to be associated with the presynaptic release of norepinephrine. Such measurements are expected to elucidate mechanisms involved in the dysregulation of the noradrenergic systems during withdrawal from alcohol and in alcohol-induced chronic organic brain syndromes.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00256-04 LCS

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.)

HPLC Methods for the Measurement of Neurotransmitters

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

PI: M. Linnoila Chief LCS, NIAAA

COOPERATING UNITS (if any)

Laboratory of Chemistry, NIADDK (K. Kirk, K. Jacobson, A. Gukowsky)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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 have developed novel chemical derivatization procedures to render biogenic amines lipid soluble and/or electroactive. The derivatives have been extracted into organic solvents and quantified with electrochemical detection. These methods have been applied to quantify serotonin, normetanephrine and phenylethylamine in human and nonhuman primate cerebrospinal fluid.

This project has been terminated.

Gusowsky F, Jacobson K, Kirk K, Marshall T, Linnoila M. A new HPLC procedure for the detection and quantification of beta-phenylethylamine, J Chromatogr 1987; 415:124-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00257-04 LCS

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.)*
Neuroendocrine Studies in Offspring of Familial Alcoholics

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

PI:	M. Linnoila	Chief	LCS, NIAAA
Others:	T. George	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*
Responses of thyroid stimulating hormone (TSH) to thyrotropin releasing hormone (TRH) have been studied in the offspring of familial alcoholics and age, sex and past alcohol exposure matched control children. Sons but not daughters of familial alcoholics were found to have exacerbated TSH responses to TRH infusions.

PROJECT DESCRIPTION:

Investigators:

M. Linnoila	Chief	LCS, NIAAA
T. George	Senior Staff Fellow	LCS, NIAAA

Objectives:

We are investigating the persistently blunted TSH response to TRH infusions in long-term abstinent alcoholics, described by Larsen and Prange, as a possible genetic marker for vulnerability to alcoholism.

Methods Employed:

In the preliminary study, fifteen sons and fifteen daughters of familial alcoholics and 30 matched control children received i.v. infusions of TRH while at bed rest in our Outpatient Clinic. Triiodothyronine, thyroxine, and TSH concentrations were quantified. The sample has been expanded to 77 subjects at the present time. We are aiming at a sample of 120 subjects to provide a definitive finding. This sample size was deemed necessary by statistical power calculations.

Major Findings:

Sons, but not daughters, of familial alcoholics had markedly higher TSH responses to TRH than their matched controls. No differences were found in prolactin growth hormone and T_3 responses between the children at risk for becoming alcoholics and controls.

Significance to Biomedical Research and the Program of the Institute:

This is potentially the first male-limited neuroendocrine abnormality described in children of familial alcoholics. If confirmed in further studies, an exacerbated TSH response to TRH infusions may become useful for identifying individuals at a high risk of becoming alcoholics. Furthermore, the finding may be indicative of a primary serotonergic deficit in boys at a high risk of becoming alcoholics.

Proposed:

We are increasing the sample size.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00258-04 LCS

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.)

Violent Behavior, Neurotransmitters, Glucose Metabolism and Alcohol Abuse

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

PI:	M. Linnoila	Chief	LCS, NIAAA
Others:	D. Goldman	Section Chief	LCS, NIAAA

COOPERATING UNITS (if any)

Department of Psychiatry, University Central Hospital, Helsinki, Finland (M. Virkkunen); IRP, NIMH (F.K. Goodwin)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	.2	.2

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

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

We have investigated neurotransmitter metabolites and glucose metabolism in incarcerated violent offenders, arsonists and healthy volunteers. We have found that low cerebrospinal fluid (CSF) 5-hydroxyindoleacetic acid (5HIAA) concentrations and hypoglycemias during oral glucose tolerance tests are associated with each other and impulsive violent acts and fire setting. In a follow-up study we found that a low blood glucose nadir and low CSF 5-HIAA concentration were powerful predictors of recidivism among impulsive violent offenders and fire setters. We are currently collecting lymphocytes from violent offenders, their family members and appropriate controls.

PROJECT DESCRIPTION:

Investigators:

M. Linnoila	Chief	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
M. Virkkunen	Senior Lecturer	University of Helsinki
F.K. Goodwin	Director	IRP, NIMH

Objectives:

To investigate psychobiological and genetic variables associated with impulsive and violent behaviors as well as alcohol abuse in humans.

Methods Employed:

Cerebrospinal fluid neurotransmitters and neurotransmitter metabolites have been quantified in samples obtained from violent offenders, arsonists and healthy volunteers. The subjects have been administered oral glucose tolerance tests and MMPIs. Careful forensic psychiatry examinations have been performed on the subjects. A follow up study on recidivism has been completed using the criminal register of Finland to detect repeat crimes. Lymphocytes are currently being collected from violent offenders and their families to be used for genetic linkage studies.

Major Findings:

Violent offenders have low cerebrospinal fluid 5HIAA concentrations compared to healthy volunteers. Arsonists have both low cerebrospinal fluid 5HIAA and 3-methoxy-4-hydroxyphenyl glycol (MHPG; the main central metabolite of norepinephrine) concentrations compared to healthy volunteers. Eleven of twenty arsonists became hypoglycemic during an oral glucose tolerance test. In a follow up study, psychobiological variables classified correctly 84% of subjects into the recidivist and nonrecidivist categories.

Significance to Biomedical Research and the Program of the Institute:

Alcohol abuse is associated with a large proportion of violent offenses and arsons. It has also been associated with a low cerebrospinal fluid 5HIAA concentration in the past. We have demonstrated clear associations between low cerebrospinal fluid 5HIAA concentration, alcohol abuse and violent behavior. Furthermore, we have found in arsonists associations between low cerebrospinal fluid 5HIAA and MHPG concentrations, abnormal glucose metabolism and alcohol abuse. These findings if replicated by others can form a rational basis for treatment interventions in these heretofore difficult to treat individuals. The recent findings on prediction of recidivism using psychobiological variables are the first to explore the feasibility of predicting dangerousness with other than behavioral means. Studies currently underway are designed to elucidate genetic contributions to impulsive and aggressive behaviors.

Proposed Course:

We are collecting lymphocytes in a new sample of subjects to relate the described findings to possible Y-chromosome abnormalities. We will enlarge our sample to blood relatives of the index individuals and appropriate controls to investigate the heredity of these conditions. We have underway a study on circadian rhythms in violent offenders because of their frequent complaint of insomnia.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00269-03 LCS

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.)
Biological Factors in Abnormal Bereavement

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

PI:	A. Roy	Visiting Associate	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA

COOPERATING UNITS (if any)
Biological Psychiatry Branch, NIMH (W. Gallucci and P. Gold)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	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 biology of bereavement, a risk period for increased drinking, was studied in a group of 19 recently bereaved subjects. Plasma cortisol and ACTH levels were measured in response to an infusion of corticotropin releasing hormone (CRH). Bereaved subjects, when compared with normal controls, had both raised basal plasma cortisol levels and blunted plasma ACTH responses to CRH. These results show that an adverse life event can cause dysregulation of the stress responsive hypothalamic-pituitary-adrenal (HPA) axis. Adverse life events are of interest as they can precipitate depression and suicidal behavior among alcoholics.

This project has been terminated.

Roy A, Gallucci W, Avgerinos P, Linnoila M, Gold P. The CRH stimulation test in bereaved subjects with and without accompanying depression, Psychiatry Research 1988;25:145-56.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00270-03 LCS

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.)
Impulsivity and pathologic gambling

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

PI:	A. Roy	Visiting Associate	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	B. Adinoff	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)
None

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NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	0

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

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

This study was conducted to investigate biological substrates of pathologial gambling. We found indices of increased central nervous system noradrenergic activity. Also, depressed gamblers showed evidence of abnormal glucose homeostasis.

PROJECT DESCRIPTION:

Investigators:

A. Roy	Visiting Associate	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
B. Adinoff	Senior Staff Fellow	LCS, NIAAA

Objectives:

To examine neurobiologic substrates of pathological gambling. Studies of other groups, who also manifest disordered impulse control have revealed evidence suggesting central serotonin deficiency and dysregualtion of glucose metabolism. Gambling is a disorder with a raised risk for alcohol abuse and there is also an increased incidence of alcoholism in families of gamblers.

Methods Employed:

Twenty gamblers were studied on our research ward and compared with 20 controls. CSF studies of monoamine metabolites and peptides were carried out. A 5-hour glucose tolerance test was performed.

Major Findings:

Gamblers had a significantly greater centrally produced fraction of CSF MHPG than controls and also showed excessive urinary excretion of norepinephrine. Depressed gamblers showed abnormality on the glucose tolerance test compared to nondepressed gamblers.

Significance to Biomedical Research and the Program of the Institute:

These data suggest that there may be neurobiologic substrates to gambling. This is of interest as gamblers are at increased risk for alcohol abuse and alcoholism.

Proposed Course:

To conduct a follow up study.

Publications:

Roy A, Adinoff B, Roehrich L, Lamparski D, Custer R, Lorenz V, Barbaccia M, Guidotti A, Costa E, Linnoila M. Pathological gambling: a psychobiological study, Arch Gen Psychiatry 1988;45:369-73.

Roy A, Custer V, Linnoila M. Depressed pathological gamblers, Acta Psych Scandinavica 1988;77:163-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00272-01 LCS

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.)*

CSF Monoamine Metabolites in Alcoholic Patients who Attempt Suicide

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

PI:	A. Roy	Visiting Associate	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	B. Adinoff	Senior Staff Fellow	LCS, NIAAA
	B. Ravitz	Medical Staff Fellow	LCS, NIAAA
	T. George	Senior Staff Fellow	LCS, NIAAA
	D. Nutt	Visiting Scientist	LCS, NIAAA
	D. Lamparski	Staff Fellow	LCS, NIAAA
	J. DeJong	Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*

None

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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.)*

Reduced cerebrospinal fluid levels of the serotonin metabolite 5-hydroxyindole-acetic acid have been reported to be commonly associated with suicidal behaviour. Alcoholics are known to often manifest suicidal behaviour. Therefore, we compared alcoholics who had (N = 18) or had not (N = 132) attempted suicide and controls (N = 29) on cerebrospinal fluid levels of monoamine metabolites. There were no significant differences among the three groups for cerebrospinal fluid levels of either 5-hydroxyindoleacetic acid, the dopamine metabolite homovanillic acid, norepinephrine, or the norepinephrine metabolite 3-methoxy-4-hydroxyphenylglycol.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION:

Investigators:

A. Roy	Visiting Associate	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
B. Adinoff	Senior Staff Fellow	LCS, NIAAA
B. Ravitz	Medical Staff Fellow	LCS, NIAAA
T. George	Senior Staff Fellow	LCS, NIAAA
D. Nutt	Visiting Scientist	LCS, NIAAA
D. Lamparski	Staff Fellow	LCS, NIAAA
J. De Jong	Staff Fellow	LCS, NIAAA

Objectives:

Diminished central serotonin turnover has been claimed to be associated with suicidal and impulsive behaviour. A high percentage of alcoholics attempt suicide at some time during their alcoholism. The risk of eventual suicide is significantly raised among alcoholics. Therefore, we decided to investigate CSF levels of the serotonin metabolite 5-hydroxyindoleacetic acid in alcoholics who have or have not attempted suicide.

Methods Employed:

We examined CSF data from the approximately 150 alcoholics who have had spinal taps on our research ward. Their data was compared with that of 29 normal controls studied on the same ward.

Major Findings:

There was no significant difference for any CSF monoamine metabolite between alcoholics who had or had not attempted suicide and controls.

Significance to Biomedical Research and the Program of the Institute:

This is the largest study to date of CSF monoamine metabolites among alcoholics who attempt suicide.

Proposed Course:

To study further biological correlates of suicidal behaviour in alcoholics.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00261-04 LCS

PERIOD COVERED

October 1, 1987 to eptember 30, 1988

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

The Pathophysiology of the Alcohol Withdrawal Syndrome

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

PI:	B. Adinoff	Senior Staff Fellow	LCS, NIAAA
Others:	M. Eckardt	Section Chief	LCS, NIAAA
	J. Rohrbaugh	Research Psychologist	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	E. Majchrowicz	Scientist Emeritus	NIAAA
	C. Marietta	Physiologist	LPPS, NIAAA
	F. Weight	Section Chief	LPPS, NIAAA
	B. Ravitz	Medical Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

Dept. of Pathology, Univ. of Texas Health Science Center, Galveston, TX (T. Jerrells); Clinical Psychobiology Branch, NIMH (L. Tamarkin).

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.75	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.)

In this project, we explore the pathophysiology of two important clinical problems frequently observed in the chronic alcoholic during withdrawal: (1) disturbed sleep-wake cycle, and (2) increased susceptibility to infection. Twenty-four hour measurements of body temperature, motor activity, ambulatory EEG, and urinary catecholamine excretion are performed on acutely withdrawing alcoholics during withdrawal. Although originally this study was designed to obtain these measurements during the first 72 hours of withdrawal, recent research from our ward indicates that this time frame should be altered. We are, therefore, evaluating biologic rhythms in 24 hours on days one, three, and eight of withdrawal. Also, plasma cortisol is obtained every half hour and plasma ACTH and melatonin is determined every hour (rather than every three hours as originally proposed) during these periods. Because of limitation on the total volume of blood samples, lying-standing norepinephrine determinations are not be obtained. Alcoholic patients and age- and sex matched controls are evaluated for general immunocompetence to determine the effects of long-term alcohol abuse and withdrawal from alcohol on the immune system. Patients are evaluated at regular intervals to investigate effects of varying durations of abstinence from alcohol on selected parameters of immunocompetence.

This project has now been completed although the sleep study is still in the process of being analysed.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION:

Investigators:

B. Adinoff	Senior Staff Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
B. Ravitz	Medical Staff Fellow	LCS, NIAAA
D. Flowers	Medical Staff Fellow	LCS, NIAAA
F. Weight	Section Chief	LPPS, NIAAA
E. Majchrowicz	Research Chemist	LPPS, NIAAA
C. Marietta	Physiologist	LPPS, NIAAA
T. Jerrells	Department of Pathology	Univ. Texas
L. Tamarkin	Research Biologist	CPB, NIMH

Objectives:

The alcohol withdrawal syndrome is characterized by pathophysiological changes in many organ systems. In this project, we explore the pathophysiology of two important clinical problems frequently observed in the actively withdrawing chronic alcoholic patient: (1) disturbed sleep-wake cycle, and (2) increased susceptibility to infections.

Methods Employed:

Upon entry into the study, patients who meet DSM-III criteria for alcohol dependence and give a positive breath sample for ethanol on admission will wear an activity monitor and a rectal temperature probe for continuous 24-hour monitoring. An indwelling venous catheter is inserted in an antecubital vein for blood sampling every 30 minutes around the clock, for determination of melatonin, cortisol, and ACTH. Monitoring and sampling procedures is conducted on the day of admission for 24 hours. All studies are repeated for 24 hours on the third and eighth days following admission. Ambulatory EEGs are worn on days two through three and seven through eight to monitor sleep EEG during withdrawal. Ambulatory EEG, body temperature, motor activity, and urinary catecholamine excretion are also obtained following three weeks of abstinence.

In the present study, we also propose to evaluate general parameters of immunocompetence in alcoholic patients and age- and sex-matched controls. Lymphocytes from peripheral blood are evaluated for their ability to respond to nonspecific mitogens, recall antigens, and allogeneic tissue antigens (mixed lymphocyte response). Alterations in the humoral immune response are evaluated by measuring antibody production in culture after stimulation of cells with polyclonal activators such as pokeweed mitogen as well as measurement of lymphokine production, which is a function of the T-cells. Lymphokines of interest include interleukin 2 and gamma interferon, both of which can be easily assayed

and yield quantitative data. Other lymphocyte functions of interest include the generation of cytotoxic lymphocytes in the mixed lymphocyte response and the measurement of natural killer cells. Of particular importance are the evaluation of relative proportions of T and B lymphocytes in the circulation and the helper and suppressor T-cell subsets, using well defined surface markers. These studies are accomplished using fluorescent-activated cell sorter techniques and address whether alterations in lymphocyte type induced by ethanol result in the immune alterations previously reported in alcoholics. Plasma obtained from lymphocyte isolation procedures is saved and stored for evaluation of antibodies reactive against the patients' lymphocytes. Fluorescent-activated cell sorting techniques are used to evaluate autoantibody reactivity.

Major Findings:

A detailed analysis of the twenty-four hour cortisol rhythms has been made on six patients. A profound disturbance of circadian cortisol rhythm was observed, with extremely high levels in some patients on day one of withdrawal. This tends to normalize over the next two weeks and all subjects were showing normal diurnal rhythmicity on the last study day (about day ten). A detailed cosinar analysis of cortisol rhythmicity in these subjects showed a significant shortening of diurnal rhythm on the first day of withdrawal. The normal twenty-four hour cycle was reduced, to as short as 13 hours in some subjects. This showed a profound disturbance of central pacemaker function in these subjects and we are now analysing the temperature and sleep data to see whether similar cycle shortenings were observed. The subjects with the most severe withdrawal showed the most pronounced cycle-shortening. These data may indicate an important contribution of the suprachiarismatic nucleus and other circadian pacemaker regions of the brain in the symptomology of alcohol withdrawal.

Significance to Biomedical Research and the Program of the Institute:

Sleep disturbance is an important clinical symptom of alcohol withdrawal. Decreased slow-wave sleep, frequent arousals, and abnormalities of rapid eye-movement (REM) sleep have been well documented during alcohol withdrawal; it has been suggested that early relapse in alcholics following abstinence may be related to REM rebound. We have demonstrated that nocturnal synthesis and release of pineal melatonin is significantly inhibited during chronic ethanol administration in the rat. It has been postulated that melatonin synthesized in the pineal is a transducer from the hypothalamic circadian pacemaker which may regulate endogenous rhythms such as the sleep-wake cycle, body temperature, and other circadian neuroendocrine rhythms such as cortisol secretion. These preliminary findings of marked stimulation of HPA axis functioning during the ethanol withdrawal syndrome are similar to those observed during severe depression, malnutrition, Cushing's syndrome, or operation stress. Signs and symptoms of ethanol withdrawal such as fatigue, weakness, hypertension, mental confusion, and depression, may be partially related to the excessive glucocorticoid levels observed during withdrawal. As cortisol levels were noted to be elevated even in the presence of a positive blood alcohol level, persons with alcohol dependence may experience elevations in cortisol on a daily basis as the ethanol level decreases (at night), contributing to long-term alterations in blood pressure, memory, immune function, mood, and stress response. The absence

of a dirunal variation in cortisol levels on the first day of withdrawal may indicate dysfunction of the circadian pacemaker, suggesting discrete impairments in neuroendocrine functioning. Twenty-four hour levels of melatonin, temperature, and activity will help clarify these alterations. In humans, excessive ethanol consumption has been associated with a number of defects in specific and nonspecific immunologic mechanisms. Control of pyogenic bacterial infections is partially dependent on the nonspecific response of polymorphonuclear leukocytes; as found in animal studies, ethanol consumption has been shown to profoundly affect moblization of these cells to sites of inflammation as well as the ability of these cells to phagocytize and kill bacteria. Other studies have shown that abnormalities in the cellular and humoral aspects of the immune system are present in alcoholics and volunteers after consumption of ethanol. These defects include an inability to respond to immunization with various antigens with a delayed-type hypersensitivity response and antibody production. This observed failure of the immune response might be due to the reported lymphopenia and depression of circulating T-cells associated with ethanol ingestion. Functional impairments in lymphocyte responses have also been reported using in vitro assays of mitogen-induced proliferation. Although the mechanisms of these impairments are unknown, it has been suggested that nutrition may play an important role. It has been reported that alcoholics show inappropriate autoimmune responses, as evidenced by the production of antibodies to small bowel epithelium and skin fibroblasts as well as a general increase in immunoglobulin levels. It has been further shown that immunization of alcoholics with a pneumococcal polysaccharide vaccine produces a significantly elevated antibody response. These data suggest that ethanol abuse might produce a selective defect in immunoregulation; in support of this hypothesis, it has been shown that alcoholic patients have an inability to generate suppressor cells in vitro. The possibility exists that one effect of ethanol on the immune system is an alteration of immunoregulatory networks leading to autoimmune responses including ethanol-induced liver disease.

Proposed Course:

This project is anticipated to continue and expand to study therapeutic interventions for the alcohol withdrawal syndrome.

The current phase is now completed, however, and data analysis will continue over the next year.

Publications:

Jerrells TR, Marietta CA, Bone G, Weight FF, Eckardt MJ. Ethanol-associated immunosuppression, Adv Biochem Psychopharmacol, in press.

Nutt D, Adinoff B, Linnoila M. Benzodiazepines in treatment of alcoholism, Recent Advances in Alcoholism, in press.

Adinoff B, Bone G, Linnoila M. Acute ethanol poisoning and the ethanol withdrawal syndrome, Med Tox, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00264-03 LCS

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.)

Sensitivity to Diazepam in Alcoholics and Children at Risk for Alcoholism

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

PI:	B. Adinoff	Senior Staff Fellow	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAA
	M. Eckardt	Section Chief	LCS, NIAAA
	E. Lane	Senior Staff Fellow	LCS, NIAAA
	R. Lister	Visiting Associate	LCS, NIAAA

COOPERATING UNITS (if any)

Neuroscience Branch, NIMH (S. Paul); GWU (H. Weingartner)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☐ (a2) Interviews

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

The benzodiazepine-GABA-chloride ionophore receptor complex has been demonstrated to be involved in the physiologic and psychologic effects of ethanol. Diazepam, a benzodiazepine, binds to this receptor complex, and demonstrates a cross-tolerance to ethanol. Recent studies have shown that diazepam-induced alterations in eye movements offer a useful measure of benzodiazepine receptor sensitivity in humans. Preliminary findings at the NIMH and NIAAA suggest an increased sensitivity to the effects of diazepam, as measured by saccadic eye movements, in alcoholics. This increased sensitivity appears to persist despite long-term abstinence (up to four years). This may suggest either long-term toxic effects of ethanol upon the benzodiazepine receptor or an alteration in the receptor that is present prior to the onset of alcohol abuse. In this study we continue our studies of diazepam sensitivity in alcoholics as well as evaluating if this increased sensitivity to diazepam is present in persons "at-risk" for the development of alcoholism compared to persons without a family history of alcoholism. Subjects will also be evaluated for EEG, ERP (event-related potentials), body sway, vigilance, tracking, memory, mood assessment and expectancy, ACTH, cortisol, prolactin, and growth hormone response to diazepam.

The first part of this protocol has been completed. A total of eight alcoholics and ten age and sex-matched controls have been assessed and the results are now being analysed. This study should be written up in the next few months and on the basis of the results a decision will be made as to whether to continue the project to study high risk subjects (children of alcoholics).

PROJECT DESCRIPTION:

Ivestigators:

M. Linnoila	Chief	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
E. Lane	Senior Staff Fellow	LCS, NIAAA
R. Lister	Visiting Associate	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
J. Stapleton	Staff Fellow	LCS, NIAAA
S. Paul	Chief	NSB, NIMH
H. Weingartner	Professor	GWU

Objectives:

To evaluate (1) benzodiazepine receptor sensitivity in persons with a history of alcohol dependence compared to normal controls, and (2) benzodiazepine receptor sensitivity in young males with an alcoholic biologic father (and non-alcoholic biologic mother) compared with young males who have no history of alcohol abuse in their family.

Methods Employed:

Alcoholics and age-matched normal controls were screened to rule out significant medical problems, psychiatric disorders, drug abuse, or history of sensitivity to benzodiazepines. Prior to being entered into the study subjects were evaluated to ascertain that saccadic eye movements were normal at steady state. On the day of the study, an intravenous catheter was placed in the forearm and diazepam was infused in doses of 25 ug/kg, 50 ug/kg, and 100 ug/kg. Each dose was infused over one minute, and doses were administered every 15 minutes. Prior to the initial dose and following each dose, subjects were evaluated for eye movement, prolactin, growth hormone, and mood. Young males (14-21 y/o) (1) with alcoholic biologic fathers and biologic mothers without a history of alcohol abuse, and (2) without a significant family history of alcohol abuse (no parents, siblings, grandparents or no more than one second degree relative with alcohol abuse) will be evaluated to rule out significant medical problems, psychiatric disorders, drug abuse, or history of sensitivity to benzodiazepines. Following a complete family history, developmental history, determination of impulsivity, and trait anxiety, subjects will partake in two infusion studies. Diazepam or placebo will be administered in random order at least two weeks apart. Doses of diazepam will be as described above, and subjects will be evaluated prior to infusion onset, after each dose, and at one and six hours after the final dose. Subjects will be evaluated for saccadic eye movements, EEG, ERP (event related potentials), body sway, vigilance, tracking, memory, mood assessment and expectancy.

Major Findings:

None

Significance to Biomedical Research and the Program of the Institute:

Ethanol and benzodiazepines (BZs) have a number of similar neuropharmacological and behavioral characteristics. Both have anxiolytic, muscle relaxant, anti-convulsant, and sedative/hypnotic properties and both exhibit cross-tolerance to one another's pharmacological effects. Clinically, the BZs are the most commonly used drug class for the treatment of the ethanol withdrawal syndrome. Therefore, it has been postulated that ethanol's effect on the BZ-GABA-chloride receptor-ionophore supramolecular complex may play a role in ethanol intoxication, tolerance, and dependence. The characterization of alterations in the sensitivity of benzodiazepine receptors in alcoholics may, therefore, help in our understanding of both the effects of chronic alcohol abuse and the etiology of alcoholism. The genetic basis for developing alcoholism has been well documented, with a point prevalence of 25% in male and 5-10% in female relatives of alcoholics compared to the general population estimates of 3-5% in men and 0.1% in women. One strategy that has been employed for identifying biological and psychophysiological characteristics that may predispose an individual to develop alcohol abuse is to determine in alcoholics those characteristics that are clinically abnormal and do not improve with abstinence, suggesting that these characteristics were abnormal prior to abusive alcohol consumption. Obviously, this strategy is based on the assumption that the consequences of alcoholism are somewhat reversible with abstinence, whereas pre-alcoholic abnormalities will not show much improvement. One example of this strategy involves the P300 component of the Event Related Potentials observed in certain cognitive paradigms. Alterations in the P300 component have been reported in detoxified adult alcoholics, and they do not return to normal with abstinence. This observation prompted Begleiter et al. to study P300 in 11 to 13 year old male children of alcoholics, resulting in the finding of reduced P300 amplitude. Our preliminary findings of possibly increased sensitivity to diazepam in alcoholics, even following long-term abstinence, suggest that such sensitivity may pre-date the onset of alcohol abuse. The determination of altered benzodiazepine sensitivity in persons at risk for the development of alcohol abuse would therefore offer 1) the potential of specific psychosocial and/or pharmacologic interventions in a clearly identified population at risk for the development of alcohol abuse, prior to the onset of the illness, 2) a possible etiology in the predisposition to the development of alcohol abuse, and 3) a further understanding of the psychopharmacologic effects of alcohol.

Proposed Course:

Eight alcoholic and age-matched normal volunteers have been evaluated. No person's "at-risk" for alcoholism or members of the respective control group have been evaluated to date.

Pubications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00231-06 LCS

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.)

Central and Peripheral Nervous System Function in Abstirent Alcoholics

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

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	B. Ravitz	Medical Staff Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	D. Flowers	Medical Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

George Washington University (H. Weingartner); Clin. Psychobiol. Br., NIMH (L. Tamarkin): Lab. of Neurorad. & Comp. Tomo., NINCDS (R. Brooks); Nuclear Medicine, CC(S. Larson). Biol. Psychiat. Br., NIMH (P. Gold).

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	2.5	2.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Behavioral deficits in alcoholics have been conceptualized in terms of two neuropathologically distinct syndromes: alcoholic dementia and Korsakoff's psychosis (alcohol amnestic disorder). Alcoholic dementia is characterized by diffuse cortical damage primarily related to the neurotoxicity of alcohol; Korsakoff's psychosis is associated with subcortical lesions due to nutritional (thiamine) deficiency. Severe memory impairment with relative sparing of other intellectual functions distinguishes Korsakoff's psychosis from alcoholic dementia (which may be clinically indistinguishable from the most common cause of dementia, Alzheimer's disease). We have recently found that sleep in Korsakoff patients is characterized by a reduced REM latency compared to normal volunteers, whereas Alzheimer patients have normal latencies. Furthermore, delta sleep is reduced in Alzheimer's disease, but is normal in Korsakoff patients. We have also demonstrated reduced daily excretion of the major urinary metabolite of melatonin, hydroxymelatonin, in patients with Korsakoff's psychosis. This finding is suggestive of impaired pineal function. Genetic differences in thiamine metabolism may predispose patients to develop Korsakoff's psychosis. Most patients with Korsakoff's psychosis whom we have studied have had a transketolase with reduced affinity for thiamine pyrophosphate. The majority of alcoholics with cognitive impairment demonstrate features characteristic of both syndromes. Pharmacologic modulation of neurotransmitter systems may be effective in treatment of the subcortical syndrome, whereas alcoholic dementia may require treatment strategies similar to those in Alzheimer's disease. This protocol is intended to utilize clinical, neuroradiological, physiological, and neuropharmacological tests to differentiate these two pathologic entities, to follow a longitudinal course, and to relate variables in treatment protocols to outcome.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION:

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
N. Salem	Section Chief	LCS, NIAAA
B. Ravitz	Medical Staff Fellow	LCS, NIAAA
D. Flowers	Medical Staff Fellow	LCS, NIAAA
R. Brooks	Research Scientist	LCS, NINCDS
P. Gold	Section Chief	BPB, NIMH
S. Larson	Chief	NM, CC
L. Tamarkin	Research Biologist	CPB, NIMH
H. Weingartner	Professor	GWU

Objectives:

Chronic organic brain syndromes due to alcoholism constitute the second most common cause of dementia in adults (approximately 10%), ranking next to senile dementia of the Alzheimer's type (40-60%). Currently, a large proportion of dementing illness can be diagnosed with certainty only by examining the microscopic structure of the brain at autopsy. The cross sectional clinical picture of alcohol-related cognitive decline may be difficult to distinguish from that of the more prevalent primary degenerative dementia (Alzheimer's disease). Chronic alcohol abuse may lead to two clinically and neuropathologically distinguishable syndromes: alcoholic dementia and alcohol amnestic syndrome (also called Korsakoff's psychosis). These two organic brain syndromes may represent extremes of the spectrum of cognitive impairments related to chronic alcoholism. Alcoholic dementia is characterized by global intellectual decline, whereas the salient clinical feature of the alcohol amnestic syndrome is a severe and persistent memory deficit with relative sparing of other intellectual functions. The majority of alcoholic patients have aspects of both syndromes; presumably the midline subcortical lesions due to thiamine deficiency may explain the amnestic component, whereas the diffuse bilateral cortical damage resulting from alcohol neurotoxicity explains the global cognitive loss. It has been postulated that polymorphisms of thiamine-requiring enzymes may influence which clinical syndrome predominates. Most of the patients with Korsakoff's psychosis in whom we have studied fibroblast transketolase have had an elevated K_M for thiamine pyrophosphate in comparison with fibroblast derived from normal controls. We have found that patients with relatively "pure" amnestic characteristics have demonstrated episodic memory impairments that resemble those found in depression and Parkinson's disease and are distinguishable from the semantic or knowledge memory deficits found in Alzheimer's disease. Furthermore, we have demonstrated significant differences in the pattern of sleep EEG

abnormalities in Korsakoff's psychosis patients compared with those with Alzheimer's disease. The sleep of Korsakoff patients resembles that of patients with depression (increased arousals and shortened REM latency). We postulate that treatment strategies directed toward modifying activation and arousal by pharmacologic modulation of neurotransmitter systems may be effective in treatment of the alcoholic amnestic syndrome. This situation is analogous to the benefits derived from pharmacotherapy in depression and Parkinson's disease, whereas alcoholic dementia requires treatment approaches similar to those in Alzheimer's disease.

Methods Employed:

We will study two groups of controls (healthy nonalcoholics and alcoholics abstinent for at least six months) and four groups of patients (detoxified alcoholics who have been abstinent from alcohol for at least one week; alcoholics withdrawn from alcohol who have been abstinent for at least three weeks; alcohol amnestic patients; and alcoholic dementia patients), using the following clinical, physiological, and neurochemical tests: 1) neuropsychological evaluation of patients to determine whether they are predominantly amnestic or demented (in collaboration with Dr. Weingartner); 2) norepinephrine response to orthostasis; 3) dose-response to norepinephrine infusion; 4) norepinephrine and endocrine responses to insulin tolerance test; 5) catecholamine and neuropeptide metabolism in cerebrospinal fluid versus plasma and urine; 6) vasopressin response to hypertonic saline infusion; 7) thyrotropin-releasing hormone and gonadotropin-releasing hormone stimulation test; 8) dexamethasone suppression test; 9) corticotropin-releasing hormone test; 10) circadian rhythms of melatonin, body temperature, and activity (in collaboration with Dr. Tamarkin); 11) sleep EEG (in collaboration with Dr. Gold); 12) therapeutic trial of the serotonin uptake blocker fluvoxamine; and 13) positron emission tomography, computerized axial tomography, and nuclear magnetic resonance imaging (in collaboration with Drs. Brooks and Larson).

Major Findings:

A number of potentially important findings have emerged. These are as follows: 1) In the saline infusion (vasopressin test) it appears that middle-aged alcoholics have blunted responses of vasopressin to the saline infusion. In other words the normal age-related increase in vasopressin response to saline is considerably less in the alcoholics. This would suggest damage to central hypothalamic mechanisms, possibly as a result of drinking, since the young alcoholics do not show a similar pattern. To be sure of this we have to increase our number of middle-aged controls (40-60 years) as we are presently doing. 2) The norepinephrine infusion data has been analysed. The previously reported blunting of response to the low rate of norepinephrine infusion was confirmed. Since plasma norepinephrine concentrations do not differ in this study between these two groups we conclude there is a subsensitivity in the alcoholics. However, the importance of this is hard to determine since with the higher rates of infusion alcoholics behave much as controls. Analysis of heart changes during the norepinephrine infusion suggests that central baroreceptor mechanisms are not altered in young alcoholics.

3) The insulin challenge test has been pursued and we have now demonstrated that amongst the alcoholic population there are two groups. One which shows enhanced responsiveness to insulin, i.e. increased medullary responses, and one which shows a blunting of this response. We are presently studying other subjects to see why such a dichotomy might exist. Analysis of the pituitary hormone responses to insulin-induced hypoglycemia is still pending. 4) Analysis of the pituitary responses to the TRH/LHRH infusion has suggested that a number of alcoholics may be borderline hypothyroid. Another batch of samples has been sent to the laboratory and these will be analysed subsequently. 5) The CRH challenge showed clearly that in alcoholics given CRH one week after withdrawal the majority had markedly blunted ACTH responses. This normalized by three weeks. Interestingly the cortisol response to the CRH challenge was normal, which is surprising given the blunted ACTH response. This may reflect adrenal hyperplasia in alcoholics, possibly secondary to the effects of withdrawal. In those subjects in which testing was performed after a long period of sobriety (up to several years in one patient); a consistent increase in ACTH responses to CRH was observed. This suggests that alcohol has a profound attenuating effect on hypothalamic responsiveness to CRH and that many years may be necessary before full return to normal function. 6) In the last report we mentioned that a number of patients with Korsakoff's syndrome showed marked memory improvement to the serotonin uptake blocker fluvoxamine. We have now initiated a prospective study titrating fluvoxamine plasma levels to optimum concentration and this should be complete within the next eighteen months.

Significance to Biomedical Research and the Program of the Institute:

Chronic organic brain syndromes due to alcoholism are responsible for approximately 10% of dementia in the adult population. The fact that only a small population of alcoholics develop complication of alcoholism suggests the importance of predisposing factors. We will attempt to identify genetic factors that may be predictive of which individuals will develop the alcohol amnestic syndromes if they abuse alcohol. We plan to develop a clinical, physiological, and biomedical classification system of alcohol-associated chronic organic brain syndromes; this system will have diagnostic, prognostic, and therapeutic applications. If the improvement previously reported with fluvoxamine can be replicated in a followup study then this will have major implications for understanding the etiology and treatment of alcohol amnestic syndrome. It of course raises the question whether other forms of amnestic disorder such as those seen in association with negative symptoms of schizophrenia might also be ameliorated by 5HT uptake blockers.

The findings of abnormal peripheral sympathetic responsiveness, especially that of the adrenal gland in young alcoholics may help us understand the damage produced by alcohol. However, they may also reflect a trait predisposition in alcoholics which could conceivably be a biological marker for the risk of alcoholism and may throw some light on the etiology of alcoholism. Clearly, we need to study nonalcoholic adult children of alcoholics to determine whether similar abnormalities exist, and thus to establish or refute whether this represents an inherited contribution to the disease. Further findings suggest that in most recently abstinent alcoholics the total ACTH response to CRH stimulation is decreased, and that this response tends to "normalize" over time. Although Z01 changes in the correlation between baseline cortisol levels

and HPA axis responses to CRH were evident even in alcoholics with greater than six months abstinence. This suggests that alterations in HPA axis functioning may persist for prolonged periods following the cessation of drinking. It is not clear from this study if these findings are due to chronic effects of ethanol or to effects of the subsequent withdrawal syndrome.

Although elevated baseline free cortisol levels were not observed in any of the alcoholic groups studied, cortisol response to CRH stimulation was proportionately greater in the one week and three week abstinent low ACTH responders compared to controls. This observation is compatible with the development of hyperplasia and hyperresponsiveness of the adrenal cortex, which is known to occur after even a few days of stimulation with exogenous ACTH or with the application of experimentally induced stress. As the group of alcoholics we tested at one and three weeks abstinence had experienced no more than mild withdrawal symptoms, were in relatively good health, and were not tested until one week following their last drink, the findings reported are most likely quite subtle compared to the changes one might observe in a poorly nourished alcoholic following delirium tremens. Our observations may, therefore, suggest that pituitary responsiveness may be severely impaired for a prolonged period of time following abstinence in more severely impaired alcoholics, and could contribute to the medical and psychological problems seen in this group.

Proposed course:

A number of components of the project have now been completed. Recruitment is taking place in the insulin, vasopressin and fluvoxamine parts as detailed in the major findings section. A detailed analysis of the CSF variables, with correlations being made with plasma and urine monoamine concentrations will be made over the next year, and these will be compared in relation to drinking history and type of alcoholism.

Publications:

Nutt DJ, Adinoff B, Ravitz B, George T, Flowers D, Eckardt M, Bone G, Martin P, Linnoila M. CSF studies in alcoholics and violent offenders, Aust Alc Drug Rev, in press.

Nutt DJ, Cowen PJ. Neurochemical studies on human volunteers, Pharmaceutical Med, in press.

Risher-Flowers D, Adinoff B, Ravitz B, Bone GHA, Martin PR, Nutt DJ, Linnoila M. Circadian rhythms of cortisol during alcohol withdrawal, Adv Alc Subst Abuse, in press.

Linnoila M, Oliver J, Adinoff B, Potter WZ. High correlations of norepinephrine, dopamine, epinephrine and their major metabolite excretion rates, Arch Gen Psychiatry, in press.

Roy A, Adinoff B, Linnoila M. Acting-out in normal volunteers: negative correlation with 5-HIAA levels, Psychiatry Res, in press.

Adinoff B. Hypothalamic-pituitary-adrenal axis functioning in recently abstinent alcoholics, In: Linnoila M, Moderator. Alcohol withdrawal and noradrenergic function. Ann Int Med 1987;107:875-89.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00249-05 LCS

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.)

Pharmacologic Reduction of Alcohol Consumption in Alcoholic Patients

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

PI:	D. George	Senior Staff Fellow	LCS, NIAAA
Others:	M. Eckardt	Section Chief	LCS, NIAAA
	R. Eskay	Research Physiologist	LCS, NIAAA
	E. Lane	Senior Staff Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	N. Salem	Section Chief	LCS, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Recent studies indicate that alcohol consumption is regulated by several interacting neurotransmitters, including the dopamine and serotonin systems. In a randomized double-blind design, chronic alcoholic outpatients will receive L-DOPA or L-5-hydroxytryptophan, both with the peripheral decaboxylase inhibitor carbidopa or placebo for a 1-year period. During this year, alcohol consumption, liver function, craving for alcohol, mental status, psychosocial functioning, and compliance with medication will be assessed at regular intervals. Prior to entry into the study, after 3 months, and at 1 year, the following procedures will be conducted to measure drug effects: (1) behavioral evaluation; (2) determination of concentrations of drugs, monoamines, hormones, and peptides in blood and cerebrospinal fluid; (3) orthostatic changes in heart rate, blood pressure, and plasma norepinephrine concentrations; and (4) assessment of plasma vasopressin response to saline infusion. Changes in alcohol consumption will be related to biochemical and behavioral parameters.

PROJECT DESCRIPTION

Investigators:

D. George	Senior Staff Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
R. Eskay	Research Physiologist	LCS, NIAAA
B. Adinoff	Senior Staff Fellow	LCS, NIAAA
E. Lane	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
N. Salem	Section Chief	LCS, NIAAA

Objectives:

Over 90% of physicians in private practice prescribe drugs for the treatment of alcoholism, although valid studies demonstrating the efficacy of pharmacotherapy in this disorder are lacking. Successful treatment outcome may be defined as one or more of the following: (1) reduction of the amount of alcohol consumed, (2) retention of the patient in treatment; (3) improvement of social and family relations; (4) maintenance of employment and financial status; and (5) amelioration of the medical and psychiatric complications of excessive alcohol comsumption. There is now considerable evidence that reduction in alcohol consumption is of fundamental importance and can be expected to decrease the frequency and severity of alcohol-induced organic disease and to favorably modify behavioral problems that lead to or result from excessive alcohol consumption. Since alcohol consumption is maintained by reinforcement, which has a neurochemical basis, it may be possible to modify drinking behavior by pharmacologic interventions that alter central neurotransmitter function. Recent studies indicate that both central dopaminergic and serotonergic mechanisms may influence ethanol consumption. Evidence for functional dopaminergic and/or serotonergic deficits in alcoholics suggest that therapeutic strategies using the dopamine precursor L-DOPA and/or the serotonin precursor 5-hydroxytryptophan may benefit some patients.

Method Employed:

Alcoholic outpatients receive L-DOPA or 5-hydroxytryptophan both with the peripheral decarboxylase inhibitor carbidopa or placebo, for a 1-year period in a randomized double-blind parallel design. Compliance is measured by pill count and determination of blood L-DOPA, L-5-hydroxytryptophan, and carbidopa levels. Alcohol consumption is monitored by a drinking logbook compiled by the patient, interview questionnaires with the patient and a significant other, sequential serum gamma GT levels, breathalyzer readings at the time of clinic visits. A lumbar puncture for measurement of biogenic amines and peptides, orthostatic norepinephrine test, and plasma vasopressin response to saline infusion prior to and after 3 months' treatment and 1 year's treatment allows neurochemical classification of patients and determination of whether patient and neurochemical characteristics are related to treatment outcome.

Major Findings:

To date, 38 patients have been admitted to protocol: 9 have successfully completed the 1-year study, and one person is actively participating. Twenty-eight have resumed drinking and/or dropped out.

Significance to Biomedical Research and the Program of the Institute:

To date, drugs to reduce alcohol consumption have not been adequately investigated, and existing studies suffer from several methodological difficulties. For example, no agent has been studied for longer than 3 to 6 months, an insufficient duration of time to determine whether the natural history of a chronic relapsing illness such as alcoholism is modified. It has been difficult to adequately measure compliance with medication and the major outcome variable of alcohol consumption. There has been no attempt to study the relationship between specific pharmacologic effects of drugs and treatment outcome. Finally, no studies have attempted to identify the neurochemical and patient characteristics which would permit a rational choice of the optimal drug for individual alcoholic patients. The design of the proposed study will allow us to address the majority of these difficulties and thereby provide information that will have direct applicability to alcoholic patients.

Proposed Course:

It is anticipated that no more subjects will be added to the project. The results are in the process of being analyzed.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00266-03 LCS

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.)

Relationship of Psychobiology to Psychopathology in Alcoholics

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

PI:	D.T. George	Senior Staff Fellow	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	A. Roy	Visiting Associate	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	D. Lamparski	Staff Fellow	LCS, NIAAA
	D. Goldman	Section Chief	LCS, NIAAA
	B. Adinoff	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS (if any)

Clinical Psychobiology, NIMH (W. Potter); Biological Psychiatry, NIMH (T. Uhde)

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Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☒ (a2) Interviews

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

Alcoholism and affective disorders frequently occur in the same individuals and in members of the same family. This association may represent the co-existence of two common disease entities in a given individual due to chance or due to (a) alcoholism resulting from self-medication of an underlying affective disorder or (b) depression resulting from toxic effects of alcohol abuse. A number of studies have attempted to unravel this complex interaction but many unanswered questions still remain. Commonly, the depression disappears within two weeks of abstinence, however, it may have a prolonged course similar to primary depressive illness. A causative relationship is inferred from studies which show an increased incidence of alcoholism in families of patients with affective disorder, a high incidence of affecive disorder in families of alcoholics and a high incidence of suicidal behavior associated with both affective disorder and alcoholism. Studies have shown that alcohol may acutely improve the sense of affective well-being but with continued intoxication this improvement may be reversed. During chronic experimental intoxication, alcoholics not only become increasingly depressed but also more anxious. Descriptive studies have shown that a large percentage of withdrawing alcoholics experience generalized anxiety and phobic reactions. In this protocol we propose to characterize certain biochemical aspects of depression and anxiety as they occur in alcoholic patients. To do this, we will examine cerebrospinal fluid and plasma for norepinephrine (lying and standing), urine for catecholamine metabolites and employ pharmacological challenge paradigms using lactate, isoproterenol and chlorimipramine.

PROJECT DESCRIPTION:

Investigators:

D. George	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
A. Roy	Visiting Associate	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
D. Lamparski	Staff Fellow	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
T. Uhde	Senior Staff Fellow	BP NIMH
W. Potter	Chief	CPB, NIMH

Objectives:

To elucidate relationships of alcoholism to other psychopathology by studying the phenomenology and neurochemical characteristics of depression and anxiety in alcoholics and their non-alcoholic family members.

Methods Employed:

Patients admitted to the study will meet DSM III criteria of the American Psychiatric Association for alcohol dependence and will have been abstinent from alcohol for at least two weeks. An examination by a psychiatrist and the Schedule for Affective Disorders and Schizophrenia (SADS) administered by a research social worker, will be used to make the diagnosis of past or present major affective disorder or anxiety disorder in alcoholic patients according to DSM III criteria. Age- and sex- matched control populations will consist of (1) normal volunteers, (2) abstinent alcoholic patients without a diagnosis of major affective disorder or anxiety and (3) adult offspring of alcoholics between the age of 18 and 75 without a diagnosis of alcoholism.

Cerebrospinal fluid will be examined for monoamines, their metabolites, and peptides. Pharmacological challenge paradigms employing isoproterenol and chlorimipramine will be used to explore respectively the adrenergic and serotonergic systems which have been implicated in depression and anxiety disorders. Lactate infusions, commonly employed to induce panic in susceptible individuals, will be used as a probe for anxiety. Chloride ion and placebo will be administered in a double blind design concurrently with lactate to observe the effects of chloride ion on anxiety.

Major Findings:

To date, 20 patients have completed the lactate infusion study (12 alcoholics with panic, 10 children of alcoholics with panic [COA]). Results showed alcoholics with panic are less likely to panic during a lactate infusion compared with children of alcoholics with panic. The panic response obtained in the COA group is consistent with that reported for panic patients in the literature. Lactate induced physiological changes (BP, pulse) as well as biochemical changes (pH, electrolytes lactate levels) showed no difference between the two groups thus yielding no obvious explanation to the reduced lactate response in alcoholics with panic. Isoproterenol and chlorimipramine infusions were admini-

stered to 20 alcoholics, 12 alcoholics with panic, 8 depressed alcoholics and 15 controls. When isoproterenol was given to alcoholics with and without panic, the group with panic showed a reduced blood pressure and heart rate response. This finding suggests a reduced Beta receptor sensitivity. Biochemical measures (norepinephrine, c AMP and isoproterenol levels) and comparison between other patient groups await analysis. Chlorimipramine infusions were analyzed for changes in prolactin, cortisol and ACTH levels. Preliminary analyses showed a trend toward increased stimulation of cortisol in depressed alcoholics compared with alcoholics without depression in response to chlorimipramine. Other patient groups and biochemical measures await further analyses.

Ten alcoholics with and without panic plus 8 COA's had lumbar punctures. Results show alcoholics with panic have statistically higher concentrations of Beta-endorphin (covaried for height, age, weight) compared to alcoholics without panic and controls. Lying/standing determinations or pulse, BP and norepinephrine levels show a trend toward reduced pulse response with standing and a concomitant increased production of norepinephrine in alcoholics with panic compared to alcoholics without panic.

Significance to Biomedical Research and the Program of the Institute:

Alcohol abuse may have an adverse affect on mood contributing to feelings of anxiety, depression and suicidal ideation. Frequently, in order to cope with these negative emotional states, the alcoholic increases his drinking which results in a compromised physical and psychological state. To date, there have been a few studies which have attempted to study the biochemical links between affective states and alcoholism. By understanding possible biochemical perturbations that arise from or contribute to alcoholism, we can gain a better understanding of the effects of alcohol and provide avenues for more effective pharmacological intervention.

Proposed Course:

In addition to the above studies we are also beginning to recruit patients into a procaine study. This examines the sensitivity of the temporal lobe and limbic system (the emotional sensor of the brain) to infusions of a drug called procaine which selectively activates them. Prediction is that alcoholics may have abnormal responses due to their chronic drinking and to the experience of withdrawal. This will be tested and a comparison will be made with controls and with patients with panic disorder.

Publications:

George DT, Nutt DJ, Walker WV, Porges SW, Adinoff B, Linnoila M. Sodium lactate and hyperventilation substantially attenuate vagal tone in normal volunteers: a possible mechanism of panic provocation? Arch Gen Psychiatry, in press.

George DT, Zerby A, Noble S, Nutt DJ. Panic attacks and alcohol withdrawal: Can subjects differentiate the symptoms? Biol Psychiatry 1988;24:240-243.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00271-02 LCS

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.)*

Pharmacological Studies in Obese Rodents

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

PI:	D. Nutt	Visiting Scientist	LCS, NIAAA
Others:	C. Gleiter	Guest Researcher	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The obese rodent provides an interesting experimental model for work in the area of alcoholism as well as obesity and diabetes in that some strains show increased alcohol preference that appears to be related to their degree of diabetes. We have begun studying several strains of obese mice in order to further characterize the pharmacological defects underlying these observations. In particular we have investigated the effect of electroconvulsive shock (ECS) on blood sugar levels since this treatment has been reported to improve glucose levels in diabetic humans. It was of interest to see whether ECS would also be an effective antidiabetic agent in this animal model prior to investigating its actions on alcohol intake. Furthermore, since abnormalities of serotonin function have been reported in alcoholics as well as in mice made diabetic by destruction of pancreatic islet cells we plan to study the actions of serotonin active drugs (eg. uptake blockers and agonists) on diabetic control, of glucose metabolism, body weight and body temperature in these anmals. These peripheral measures will be correlated with indices of central serotonin functions.

PROJECT DESCRIPTION

Investigators:

D. Nutt	Visiting Scientist	LCS, NIAAA
C. Gleiter	Visiting Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA

Objectives:

To investigate the pharmacology of the obese rodent in relationship to its alcohol preference, obesity and diabetes. in particular we plan to explore the mechanisms by which elecroconvulsive shock (ECS) may ameliorate plasma glucose control in these animals. We shall use measures of body weight, body temperature and plasma glucose to follow the action of ECS on diabetic control. Subsequently we shall measure brain receptor binding and brain concentrations of several neurotransmitters thought to be of importance in glucose metabolism, namely serotonin and norepinephrine. We also plan to see whether drugs which act on these transmitters have effects similar to ECS on diabetic control. We shall administer, on a chronic basis, several serotonin and norepinephrine uptake blockers as well as pre and postsynaptic receptor agonists and antagonists to determine if they have effects on diabetic control similar to that of ECS. If they do then we shall follow up these observations by measuring central neurotransmitter function using receptor binding assays as well as by measuring brain concentrations of transmitters and metabolites. We also plan to study the effects of blood glucose levels and the above treatments on receptors that may control food intake using receptor binding of drugs such as the appetite suppressant mazindol.

Methods Employed:

The initial studies were made in obese mice and because the results were interesting we shall extend them to obese rats. Blood glucose levels are assessed using a commerical glucometer which requires only a single drop of blood from the tail. Electroconvulsive shock is administered using ear clip electrodes and results in an immediate seizure with full amnesia. Brain receptor and neurochemical studies will be carried out using standard assay methods. If preliminary results suggest an important action of ECS on blood glucose then we shall implant indwelling cannulae into the jugular vein of obese rats to enable us to make daily measurements of plasma hormones such as insulin. The obese rodent provides an interesting experimental model for work in the area of alcoholism as well as obesity and diabetes in that some strains show increased alcohol preference that appears to be related to their degree of diabetes. We have begun studying several strains of obese mice in order to further chacterize the pharmacological defects underlying these observations. Since abnormalities of serotonin function have been reported in alcoholics as

well as in mice made diabetic by destruction of pancreatic islet cells we plan to study the actions of serotonin active drugs (eg. uptake blockers and agonists) on diabetic control, body weight and body temperature in these animals. These peripheral measures will be correlated with indices of central function.

Major Findings:

At present we have verified that glucose levels in obese mice with diabetes resemble those of humans with the maturity onset type of diabetes. Furthermore, ECS normalized blood glucose concentrations in diabetic obese mice. Less effect was noted in those mice that have insulin deficient diabetes (similar to the human juvenile onset type). The improvement in blood glucose in the obese mice was not due to major changes in body weight or body temperature. It occurred after only a single seizure and persisted for several weeks. Blood glucose levels in the nondiabetic animals did not change significantly over the course of treatment.

Significance to Biomedical Research and the Program of the Institute:

These findings may help us understand the mechanisms of action of ECS and drugs which could be of value in treating diabetes. Furthermore, since the obese mice have a predilection to consume ethanol, studies on them may elucidate the control of ethanol preference in mice. If we can determine the effects of our treatments on ethanol preference in these obese animals we may gain insights into the control of drinking behavior and possible means of manipulating it.

Proposed course:

So far we have studied about 50 obese animals. We plan to continue the ECS studies and begin exploring the actions of the serotonin uptake blockers in these mice. In addition we shall start the more detailed assessments of plasma hormone levels following ECS in the obese diabetic rat.

Publications:

Nutt DJ, Gleiter CH, Linnoila M. Repeated electroconvulsive shock normalizes blood glucose levels in genetically obese mice (C57BL/6J ob/ob) but not in genetically diabetic mice (C57BL/KSJ db/db), Brain Res 1988;448:377-80.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00260 04 LCS

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 Social Drinking on Blood Pressure

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

PI:	B. Ravitz	Medical Staff Fellow	LCS, NIAAA
Others:	R. Eskay	Research Physiologist	LCS, NIAAA
	J. Karanian	Senior Staff Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	N. Salem	Section Chief	LCS, NIAAA
	G. Bone	Guest Researcher	LCS, NIAAA

COOPERATING UNITS (If any)

Hypertension-Endocrine Branch, NHLBI (H. Keiser)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☒ (a2) Interviews

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

Hypertension is common in the adult population of the United States. It has been demonstrated to be associated with increased cardiovascular morbidity and mortality. Alcohol consumption is also prevalent and may play an important causative or contributory role to elevate blood pressure in up to one-third of all hypertensives. The association between hypertension and alcohol consumption awaits causative explanation. Elucidation of the pathophysiology of the alcohol associated increment in blood pressure is the purpose of this study. Blood pressure is measured using a 24-hour ambulatory monitoring system for several days in normotensive and hypertensive social drinkers during periods of usual alcohol consumption and abstinence. Subjects are on a low monoamine diet for the duration of the study. Blood and urine samples are obtained during baseline or usual alcohol consumption, abstinence, and return to baseline levels of alcohol intake for measurement of neurotransmitters, neuromodulators, and electrolytes involved in blood pressure regulation. Changes in these regulatory systems are related to blood pressure in the three phases of the study.

PROJECT DESCRIPTION:

Investigators:

B. Ravitz	Medical Staff Fellow	LCS, NIAAA
R. Eskay	Research Physiologist	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
N. Salem	Section Chief	LCS, NIAAA
G. Bone	Guest Researcher	LCS, NIAAA
H. Keiser	Chief	HEB, NHLBI

Objectives:

The association between alcoholism and hypertension was first observed in 1915 among French Legionnaires. Over the last 15 years, many population studies have suggested that consumption of ethanol is associated with an increased prevalence of systolic and diastolic hypertension. This is most noticeable in those persons consuming three or more drinks per day. This relationship is independent of weight, tobacco use, stress, age, physical activity, and caffeine consumption. An ongoing controversy exists as to whether elevated blood pressure is a primary effect of ethanol or is due to the ethanol withdrawal syndrome. In favor of it being a primary effect is the linear relationship between alcohol intake and blood pressure, the fall in blood pressure with abstinence, and the return of hypertension with reintroduction of alcohol, all of which have been previously reported.

The mechanism of the relationship between alcohol consumption and blood pressure regulation is unknown. The elevated blood pressure in chronic alcoholics does not appear to be explained by abnormalities in the renin-angiotensin system, catecholamines, or cortisol. This study is designed to determine the effect of moderate ethanol intake and abstinence from ethanol on blood pressure monitored continuously in the subjects' natural environment and to elucidate the mechanisms underlying the blood pressure changes. Subjects will serve as their own controls during abstinence.

Methods Employed:

We will study normotensive and hypertensive men 21 years of age or older who have at least a one-month history of average daily alcohol intake of 45-90 grams, and do not meet DSM-III criteria for alcohol abuse. Subjects must have had a previous period of abstinence without serious withdrawal symptoms and be in good health with no significant abnormalities on clinical examination other than an elevated blood pressure. Subjects whose systolic and diastolic blood pressures are less than 145 mm Hg and 90 mm Hg, respectively, will be considered to be normal volunteers; subjects with blood pressures greater than this will be considered hypertensive.

All subjects will follow a low monoamine diet for the duration for the study. Subjects will be asked to continue their usual amount of alcohol consumption (45-90 grams of alcohol per day) while wearing ambulatory blood pressure and

activity monitors continuously for 48 hours. Three consecutive 24-hour urines will be collected for measurement of Na, K, Cl, Ca, creatinine clearance, cortisol, and catecholamine metabolite excretion rates. During the first two weeks of outpatient observation, subjects will monitor and record their own blood alcohol levels (BAL), using a portable hand-held breathalyzer, hourly after the first drink of the day until BAL returns to zero or the subject goes to sleep, and on awakening in the morning.

On the days of blood pressure monitoring, subjects will come to the Clinical Center at 8 AM and 4 PM. On arrival, a double stop-cocked i.v. line will be placed for blood sampling and BP will be measured by a Dynamapp automatic blood pressure monitor. After the subjects rest for two hours, blood will be withdrawn for homovanillic acid, norepinephrine, 3-methoxy-4-hydroxyphenylglycol, serotonin, epinephrine, vasporessin, adrenocorticotropin (ACTH), atrial natriuretic factor (ANF), prostacyclin, thromboxane, renin, angiotensin, magesium, ionized calcium, and creatinine concentrations. Immediately after the blood sample is obtained, subjects will stand and blood pressure will be measured every minute for five minutes; at the fifth minute, blood will be drawn again for quantification of catecholamines, ANF, and vasopressin. Subjects will then be asked to refrain completely from alcohol use for two weeks. Blood pressure and activity will be monitored for the first 72 hours and the last 48 hours of those weeks, and urine and blood samples will be obtained as in the first part of the study. Following this abstinence period, subjects will once again return to their usual ethanol intake. Blood pressure and activity will be monitored continuously for the first 72 hours and then two weeks later for another 48 hours. Breathalyzer measurements will be made daily from the onset of drinking until the final blood pressure measurement is obtained two weeks later. Blood and urine will be studied when blood pressure is being monitored, as in the first part of the study.

Major Findings:

Ten normotensive individuals have been studied and the data are currently being analyzed. Two weeks of moderate ethanol intake does not cause hypertension in normotensive individuals. Resumption of drinking following a two week abstinence produces a significant fall in both diastolic and systolic BP with a rise in heart rate. A significant increase in blood pressure is seen after two weeks drinking as compared with the first three days. The mild pressor effect of ethanol is seen only in the morning when blood alcohol levels are zero. We are collecting the data on hypertensive social drinkers and completing additional studies on normotensive subjects.

This study is now complete, in that we have studied 14 normotensive heavy social drinkers. The data is being analysed. The analyses should be complete within the next few months. An interim analysis of the data suggests a significant increase in blood pressure after two weeks of drinking as compared with the first three days. In addition we found a pressor effect of ethanol in the mornings, only when blood alcohol levels were zero. This suggested the withdrawal phenomena may contribute to alcohol-induced hypertension and this possiblility is now being more thoroughly analysed.

Significance to Biomedical Research and the Program of the Institute:

In the Framingham study, 20% of the population had blood pressures greater than 160/95 mm Hg while 45% had values in excess of 140/90 mm Hg. The increased cardiovascular morbidity and mortality associated with hypertension has been well documented. Alcohol consumption is prevalent in the adult population: approximately 30% consume more than four drinks (approximately 60 grams) per week. Alcohol consumption may play an important causative or contributory role to elevated blood pressure in 5-30% of hypertensive persons. The causal mechanism for the association between hypertension and alcohol consumption awaits an explanation. Information derived from this study will directly address this issue.

Proposed Course:

The project will continue for approximately two years.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00265-03 LCS

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 Alprazolam, Diazepam, Clonidine, and Placebo upon Ethanol Withdrawal

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

PI:	B. Ravitz	Medical Staff Fellow	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	E. Lane	Senior Staff Fellow	LCS, NIAAA
	D. Flowers	Medical Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The ethanol withdrawal syndrome, which is partially characterized by an increased activity of the noradrenergic system, is at present most commonly treated with diazepam or chlordiazepoxide, both conventional benzodiazepines. Alprazolam, a new benzodiazepine, has been demonstrated to be successful in the pharmacotherapy of depression and anxiety disorders, in contrast to the conventional benzodiazepines. Alprazolam may have a particularly potent inhibitory action on the noradrenergic system. It can, therefore, be postulated that alprazolam may be an effective and specific treatment for the ethanol withdrawal syndrome. Clonidine, a conventional antihypertensive, has been used to successfully treat withdrawal from the opiates, and most recently, nicotine and alcohol. This study will compare the effects of alprazolam, clonidine, diazepam, and placebo on: 1) the signs and symptoms of the ethanol withdrawal syndrome, and 2) the noradrenergic overactivity of the ethanol withdrawal syndrome. Noradrenergic activity will be evaluated by determinations of cerebrospinal fluid (CSF), and plasma, catecholamines and their metabolites, plasma norepinephrine laying and standing, and lymphocyte B-adrenergic receptor sites. Also, changes in CSF pH have been reported during ethanol withdrawal. We will, therefore, evaluate CSF pH both during acute withdrawal and following three weeks of abstinence.

PROJECT DESCRIPTION:

Investigators:

M. Linnoila	Chief	LCS, NIAAA
E. Lane	Senior Staff Fellow	LCS, NIAAA
B. Ravitz	Medical Staff Fellow	LCS, NIAAA
D. Flowers	Medical Staff Fellow	LCS, NIAAA

Objectives:

To evaluate: 1) Clinical efficacy of diazepam, clonidine, and alprazolam compared to placebo in the treatment of the signs and symptoms of the ethanol withdrawal syndrome, 2) noradrenergic overactivity observed during ethanol withdrawal, and 3) differential effects of diazepam, clonidine, and alprazolam upon the noradrenergic overactivity during ethanol withdrawal. In addition, determinations of the changes in CSF pH during ethanol withdrawal will offer a better understanding of neurophysiologic alterations that occur during the ethanol withdrawal syndrome.

Methods Employed:

Upon admission to 3B-N, patients are evaluated hourly for: 1) signs and symptoms of withdrawal with the CIWA test, 2) blood pressure and pulse, 3) breath alcohol level. An intravenous catheter with heparin lock is inserted upon admission to the ward. CBC and electrolytes are obtained immediately upon admission and the results are evaluated prior to the onset of the study. When the patient demonstrates a significant withdrawal syndrome, the study is initiated. Patients are requested to remain supine for one hour. Following one hour of bedrest, 55 milliliters of blood are withdrawn for catecholamines and lymphocyte B-adrenergic receptors. The subject then stands for five minutes, during which time blood pressure and pulse are automatically obtained once every minute. Following five minutes of standing, five milliliters of blood is withdrawn for catecholamines. The patient is then requested to lie down again for an additional hour. Twenty-three milliliters of blood is obtained for catecholamines, neuropeptides, glucose, and electrolytes. A lumbar puncture is performed. CSF is analyzed for catecholamines, indolamines, neuropeptides, alkaloid condensation products, pH and prostaglandins. Following the lumbar puncture, the patient is requested to remain at bedrest for six hours, and pharmacologic treatment for the alcohol withdrawal begins. Patients are randomly assigned to be administered either diazepam, alprazolam, clonidine, or placebo. Assignments are made by pharmacy and patient assignment is unknown to ward staff and physicians. Patients are administered drug in a loading dose design. Medications are administered ql hr. until the signs and symptoms of alcohol withdrawal subside (CIWA < 10). The doses are as follows: alprazolam 1 mg po q 1 hour; diazepam 10 mg po q one hour; clonidine mg q 2 hours (the off hour will be a placebo). No more than 12 doses are administered on an hourly basis. Drugs are administered on subsequent days every 6 hours as needed if withdrawal symptoms recur. Blood pressure, pulse, and hydration status are closely monitored.

If at any time during the study the subject appears to be in progressive, severe withdrawal (CIWA > 25, consistent blood pressure of systolic > 70 mmHg and/or diastolic > 110 mmHg) and does not respond to the assigned medication, the blind is broken and the subject is treated with the appropriate medications. CBC and electrolytes are again be obtained on the second day of hospitalization prior to continuation of the study.

On the second, third, fourth, and seventh day following the onset of alcohol withdrawal, blood samples for catecholamines, lymphocyte B-adrenergic receptors, and lying/standing norepinephrine are obtained as above. All studies, including lumbar puncture, are repeated in the third week of hospitalization. Five milliliters of blood are obtained just prior to each dose (except the first) and every six hours for thirty-six hours after the last dose for plasma drug levels. Twenty-four hour urine collections, quantification of activity with activity monitors, and mood rating scales are also be obtained.

Major Findings:

Project is in its initial start-up phase.

Significance to Biomedical Research and the Program of the Institute:

The ethanol withdrawal syndrome is accompanied by signs of sympathetic nervous system overactivity, such as sweating, tachycardia, hypertension, and tremor. Increased levels of 3-methoxy-4-hydroxyphenylglycol (MHPG), the central metabolite of norepinephrine, have been reported in cerebrospinal fluid (CSF) and blood of alcoholics during prolonged alcohol withdrawal. A significant positive correlation of MHPG concentrations in CSF with heart rate, systolic and diastolic blood pressure, tremor, anorexia, and sweating (all common signs and symptoms of alcohol withdrawal) has been demonstrated in alcoholics during alcohol withdawal. Levels of CSF norepinephrine are also increased during alcohol withdrawal, and the levels of norepinephrine and MHPG in the CSF decrease in parallel with the resolution of the signs of alcohol withdrawal. These studies strongly suggest that medications which decrease noradrenergic overactivity may be successful in ameliorating the signs and symptoms of alcohol withdrawal.

In North America, benzodiazepines are considered the treatment of choice for ethanol withdrawal. Of these, diazepam and chlordiazepoxide are still the most commonly used. Although diazepam and chlordiazepoxide are reasonably effective in controlling acute withdrawal states, their long half-lives and pharmacokinetics may result in persistence of toxic effects beyond the requirement for sedation. Patients requiring large amounts of diazepam or chlordiazepoxide to control severe withdrawal states often remain lethargic, ataxic, or confused for several days after the withdrawal state has resolved. Toxic effects may be accentuated in patients with severe hepatic dysfunction and in the elderly. Large doses of diazepam or chlordiazepoxide may cause respiratory decompensation in patients with obstructive pulmonary disease, a common complication in older alcoholics. Also, neither diazepam nor chlordiazepoxide have antidepressant effects beyond the reduction of depression-associated anxiety.

Alprazolam, a triazolobenzodiazepine, is a more potent anxiolytic and has less toxicity than diazepam. It has been suggested that alprazolam may have a more specific effect upon the noradrenergic system than diazepam. Alprazolam has also been reported to have antidepressant properties equivalent to imipramine, and to alter REM sleep parameters and CSF norepinephrine and serotonin in the same manner as classical antidepressants. Alprazolam, thus, comes close to the theoretical profile of an ideal drug for managing acute ethanol withdrawal states: (1) a possible specific inhibitory action upon the noradrenergic overactivity of ethanol withdrawal; (2) prompt onset of sedation following oral administration to achieve rapid intervention in the evolving withdrawal state; (3) relatively short half-life to permit rapid metabolism and excretion of the drug to minimize persistence of toxic effects; (4) little or no biological activity of intermediate metabolites; and (5) antidepressant effect to facilitate motivation for postwithdrawal rehabilitation efforts. These theoretical advantages have, however, not been tested in controlled clinical trials on treatment of alcohol withdrawal.

Clonidine, a specific $alpha_2$-adrenergic agonist, is a commonly used antihypertensive. It is also frequently used for the treatment of opiate withdrawal. Recent studies indicate that clonidine may also be beneficial in the treatment of withdrawal from nicotine and alcohol. Because of its side effects profile, clonidine may offer a superior pharmacologic treatment in the management of acute ethanol withdrawal. The effect of clonidine on the noradrenergic overactivity of ethanol withdrawal has not been evaluated. In addition to further elucidation of the noradrenergic overactivity of the ethanol withdrawal syndrome, this study will help determine if alprazolam or clonidine is a safer, more specific, and more effective treatment for the ethanol withdrawal syndrome.

Proposed Course:

This project will continue for approximately two years. Recruitment to this study has been slow due to the precise requirements needed for subjects and admission times. Four subjects have been put through the study in the last year and we hope to accelerate recruiting in the next few months.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00233-06 LCS

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.)

Family Studies of Alcoholism

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

PI:	D. Lamparski	Staff Fellow	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	V. Moore	Social Worker	LCS, NIAAA
	D. Garnett	Social Worker	LCS, NIAAA
	A. Roy	Visiting Associate	LCS, NIAAA
	D. Goldman	Section Chief	LCS, NIAAA
	D. Nutt	Visiting Scientist	LCS, NIAAA
	D. Spiegler	Research Psychologist	DBE, NIAAA

COOPERATING UNITS (if any)

Social Work Department, Clinical Center, NIH (D. Rooney)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science, Unit of Family Studies

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	4.0	.

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 Unit of Family Studies has two major functions (1) to recruit and assess alcoholics, controls and their families, for various investigators within the Laboratory of Clinical Studies; and (2) to conduct psychosocial studies of alcoholic families and their individual members. In the current year, Unit staff have focused on coding and entering onto a computer the data collected since the inception of the Laboratory. A series of correlational studies comparing different subtypes of alcoholics are underway. A preliminary study comparing suicidal versus non-suicidal alcoholics on clinical, psychosocial and family variables has been carried out. In addition, the Unit has begun a study examining middle class black alcoholic families. Unit staff have also been collaborating with the Unit on Genetic Studies in identifying and phenotyping several pedigrees for linkage analysis.

PROJECT DESCRIPTION:

Investigators:

D. Lamparski	Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
V. Moore	Social Worker	LCS, NIAAA
D. Garnett	Social Worker	LCS, NIAAA
A. Roy	Visiting Associate	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
D. Nutt	Visiting Scientist	LCS, NIAAA
D. Spiegler	Research Psychologist	DBE, NIAAA
D. Rooney	Social Worker	CC, NIH

Objectives:

To accumulate and evaluate clinical and genetic data through comparative studies of alcoholic and normal control patients and their respective family members. To investigate the role of genetic and environmental influences through: (1) studies of multigenerational families, family process, and family systems, and their relation to the maintenance of abusive and addictive drinking behavior; (2) phenomenological studies of subgroups of alcoholics (women, blacks, and Hispanics); (3) comparisons between different alcoholic populations (i.e., those who attempt suicide vs. those who do not; those who become violent while drinking vs. those who do not); (4) longitudinal studies of children at risk, focusing on predictive factors and early detection; and (5) systematic studies of response to behavioral and psychotherapeutic intervention.

Methods Employed:

Assessment and data collection methodology includes use of the following: SADS-L, RDC-F, MMPI, Michigan Alcoholism Screening Test, Faces-III (Olsen) and Locke-Wallace Marital Adjustment Scale, as well as other instruments which will enable us to quantify psychosocial characteristics.

Major Findings:

We compared two different systems which categorize individuals into Type 1 and Type 2 alcoholics. The two systems gave quite disparate classifications in a sample of 104 men with no history of major psychiatric disorder. In von Knorring's system, 46% of the alcoholics were classified as Type 1 and 54% as Type 2 whereas in Cloninger's system, 4% were classified as Type 1, 20% as Type 2, 4% met neither Type 1 or Type 2 criteria and 69% met both Type 1 and Type 2 criteria. The two systems were concordant in identifying 14 Type 1 and 4 Type 2 alcoholics. They were discordant in classifying the remaining 86 alcoholics. While von Knorring and Cloninger use the same terminology, the lack of congruity between the two sets of criteria may produce considerable confusion in future biological research. We are in the process of developing and validating alternative criteria for differentiating the two types of alcoholics.

Significance to Biomedical Research and the Program of the Institute:

The long-term focus of the studies is on a better understanding of the interplay between genetic factors and environmental events, especially dysfunctional patterns of family interaction. The continued availability of graduate students and guest research investigators, and the training of staff in computer skills enhance the Unit's future research capabilities. It is anticipated that the analysis of our demographic and clinical interview data will determine additional research directions.

Publications:

Mathiasen EH, Davenport YB. Reciprocal depression in recovering alcoholic couples: The efficacy of psychodynamic group treatment, Group 1987;12:45-55.

Mathiasen EH, Davenport YB. Couples psychotherapy group treatment of the 11 married alcoholic, Group, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00234 06 LCS

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 Genetic Studies of Alcoholism

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

PI:	D. Goldman	Section Chief	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	R. Lister	Visiting Associate	LCS, NIAAA
	C. Rajagopal	Visiting Fellow	LCS, NIAAA
	R. Limson	Visiting Fellow	LCS, NIAAA
	J. Stoll	Guest Researcher	LCS, NIAAA
	W. Chen	Senior Staff Fellow	LCS, NIAAA
	R. Cotton	Guest Researcher	LCS, NIAAA

COOPERATING UNITS (if any)
Laboratory of Viral Carcinogenesis, NCI (S. O'Brien); VA Medical Center, Portland, OR (J. Crabbe); Program Resources Incorporated, Frederick, MD (M. Dean).

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section on Genetic Studies

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10	6	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.)

To identify unknown genetic loci determining alcoholism, we are testing for linkage or association between genetic markers and behavioral phenotypes. The probability of establishing linkage or association is being maximized by 1) focusing on alcoholism with impulsivity/aggressivity as a prominent accompanying behavioral trait, 2) utilizing mouse genetic models, 3) using very large panels of DNA and protein polymorphisms and 4) studying in detail candidate genetic loci including tryptophan hydroxylase, the alcohol dehydrogenases and Y chromosome loci. Human linkage markers include 390 DNA probes of which we are currently typing 100 and also include more than 50 polymorphic proteins detectable by two dimensional protein electrophoresis (2DE). These polymorphisms are being typed in two large families with alcoholism. In the mouse, we identified 14 brain polypeptide variants and preliminarily mapped a locus for alcohol preference to chromosome 1. We cloned, sequenced and analyzed the expression of human Class III alcohol dehydrogenase. We detected a Sac 1 RFLP for Class III ADH and established that it is Class III HDH in a wide variety of tissues and in the fetus with a constant message size. We are determining whether Class III ADH is part of the ADH gene complex which we have demonstrated is present on Chromosome 4. We have cloned, sequenced and analyzed the expression of a mouse mastocytoma tryptophan hydroxylase.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION:

Investigators:

D. Goldman	Section Chief	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
R. Lister	Visiting Associate	LCS, NIAAA
C. Rajagopal	Visiting Fellow	LCS, NIAAA
J. Stoll	Staff Fellow	LCS, NIAAA
W. Chen	Staff Fellow	LCS, NIAAA
R. Cotton	Guest Researcher	LCS, NIAAA
P.R. Giri	Visiting Associate	LPPS, NIAAA
S. O'Brien	Chief	LVC, NCI
J. Crabbe	Staff Scientist	VA Med. Ctr., Portland, OR
M. Dean	Staff Scientist	PRI,Frederick, MD

Objectives:

The effects of the multiple genetic and environmental factors which cause alcoholism will generally be observable only in combination. Strategically, it is advantageous to study families because this reduces environmental and genetic heterogeneities. In addition, inbred and outbred mouse models can be used to study particular components in depth. Molecular genetic methods offer the possibility of providing markers for the genetic determinants and for elucidating the roles of candidate genetic loci.

To maximize the extent of genetic homogeneity, we are studying large families also and individual males showing alcoholism associated with impulsivity/aggression. We have chosen this particular subtype of alcoholism (Type II) as a focus because: 1) studies done elsewhere indicate alcoholism associated with impulsivity/aggression is genetically transmitted, 2) the impulsive/aggressive phenotype may be, in both humans and animals, associated with lower central serotonergic activity. Also, the finding of extra or abnormally long Y-chromosomes in males with low central serotonergic activity provides a possible connection to the greater prevalence of alcoholism in males than in females.

The main objectives of these studies are: 1) To use DNA and protein polymorphisms in combination with human families and mouse strains to locate genes responsible for alcoholism-associated behavioral differences and, 2) To clone and study the molecular biology of tryptophan hydroxylase and Class III ADH, which are candidate genetic loci for alcoholism.

Methods employed:

Linkage studies in Human Families

Familial alcoholics and their families are studied in collaboration with the Unit on Family Studies (LCS, NIAAA), the Section of Clinical Brain Research (LCS, NIAAA) and Lynn Goldin (NIMH). Members of the Unit on Family Studies administer subjects the Schedule for Affective Disease and Schizophrenia (SADS)

diagnostic interview, a structured family history and a number of psychological tests including the MMPI (which yields subscale data normative for impulsivity), Matching Familiar Figures Test (MFFT), Tridimensional Personality Questionnaire (TPQ), and Eysenck Personality Questionnaire (EPQ).

For DNA and protein genetic markers, a blood sample is obtained for the establishment of a lymphoblastoid cell line. Cells are transformed using Epstein-Barr virus and cultivated to provide sufficient DNA and protein for marker studies. Cells are cryopreserved in liquid nitrogen.

The genetic linkage method depends on demonstrating non-random genetic assortment of two genetic traits. This occurs when the two traits are determined by chromosomal loci which lie close enough to each other so that they tend not to be separated by recombination. Computer programs including LIPED and new multipoint linkage programs are used to perform tests for linkage under different models of transmission. A collaborator of ours, Dr. Lynn Goldin (NIMH), has extensive experience with these programs.

The fraction of the genome which can be analyzed by the linkage method depends on the number of polymorphic markers which are available because the markers must usually lie within 10-20 centimorgans of the locus to be mapped.[3,6] Polymorphisms are genetic variants with an allelic frequency of greater than 1%. They can be used as genetic linkage markers if they are found to be appropriately transmitted in a family of interest or if they show an association in a population (linkage disequilibrium). We type more than 50 protein polymorphisms by 2DE, in which erythrocytes, serum and lymphoblasts are separated by isoelectric point in the first stage and size in the second stage. These polymorphisms appear as variants in isoelectric point.[6]

The DNA polymorphisms are of two types. Restriction Fragment Length Polymorphisms (RFLP) are detected by isolating DNA, cutting it with a sequence-specific enzyme, size-separating the fragments, denaturing and transferring the fragments to nitrocellulose and visualizing specific fragments by hybridizing a specific cloned fragment which has been labeled. Variable Number of Tandem Repeats (VNTRS) are polymorphisms involving a variable number of copies of a single sequence which are repeated in a tandem array. In collaboration with Stephen O'Brien (NCI, Laboratory of Viral Carcinogenesis) and Michael Dean (Program Resources Inc., Frederick, MD), we are currently typing 100 RFLPs and have a large collection of probes which recognize VNTRs.

Molecular Cloning and Analysis of Candidate Loci

In molecular cloning studies involving Class III ADH and tryptophan hydroxylase, we have made cDNA libraries in lambda phage starting from mRNA and have also used commercially available CDNA libraries. Libraries have been screened for relevant cDNA clones using oligonucleotide probes synthesized by us. In the case of the mouse mastocytoma tryptophan hydroxylase we cloned, we prepared a cDNA library and screened it with oligonucleotides specific to a putative tryptophan hydroxylase previously isolated from rat pineal. In the case of Class III ADH, we first purified the protein, then we sequenced several of the peptides and screened a commercially available human liver cDNA library.

For expression studies, the "Northern" blot methodology is used: mRNA is extracted from tissues, electrophoresed on agarose gels, transferred to a membrane and hybridized to a specific probe which recognizes a specific RNA or several specific RNA message sizes.

For mapping these genes in humans and mice, genomic DNA from hybrid cell lines is digested with restriction enzymes and hybridized with a probe to reveal a DNA fragment characteristic of the species in which the gene is to be mapped. By using a panel of hybrid cell lines containing different combinations of chromosomes, it is possible to map the gene to a specific chromosome.

To study the relationship between closely positioned genes such as the class I ADH genes, we have used pulsed field gradient electrophoresis. In this technique, large DNA fragments are generated by cutting genomic DNA with restriction enzymes which recognize rare sites. The restriction enzyme digestions are performed in agarose blocks to protect the DNA from shearing and the resulting large fragments are separated using an alternating (pulsed) electrical field in agarose gels. In this way large fragments of DNA (> 1 million bases) can be resolved.

Major Findings:

Linkage Studies in Mice

Using the genetic linkage approach and a panel of 14 brain polymorphic protein markers detected by two-dimensional protein electrophoresis and described by us[1], a possible locus controlling ethanol intake (LTW-4) was mapped to mouse chromosome 1.[4] Ethanol intake (preference) was measured in collaboration with Richard Lister (Clinical Brain Research) and John Crabbe (VA Medical Center, Portland). The association was found using two approaches. The first involved the BxD recombinant inbred mouse strains, which are widely used in genetic mapping. The BxD strains were generated by crossing C57/Bl6 and DBA/2J parental strains so that their chromosomes are mosaics of DNA segments from these two strains. Linkage between genes can be detected by searching for nonrandom association of two genotypes in the BxD strains. We found that low intake BxD strains significantly more often showed the acidic LTW-4 phenotype. In a second experiment, 19 less closely related inbred strains were typed and again a significant association was found between low ethanol preference and the acidic variant of LTW-4.[4] Additional experiments are underway to investigate this association.

Linkage Studies in Humans

Two large alcoholism families have been clinically characterized, and lymphoblast cell lines established and DNA extracted from family members. Additional families are being studied.

Regarding DNA polymorphisms, due to a collaborative effort with S. O'Brien (laboratory of Viral Carçinogenesis, NCI) and M. Dean (Program Resources Inc., Frederick, MD), we are now typing 100 restriction fragment length polymorphisms in the alcoholism families. Due to the efforts of M. Dean, we have collected

290 additional DNA probes, giving us a total of 390 and a minimum of 5 DNA polymorphisms for each human chromosome. Included in this collection are 90 highly polymorphic VNTR probes. Because of the 2-3/1 ratio of alcoholism in males as compared to females, three highly informative Y chromosomal DNA polymorphisms are being typed in alcoholic males to search for population linkage disequilibrium with a specific Y chromosomal haplotype. All of the 390 probes detect polymorphisms having an information content of at least 0.20, so that they frequently segregate in our families and are useful for linkage. The collection is managed with the help of a microcomputer-based database. These polymorphisms are also highly informative for population and phylogenetic analyses.[2,9]

Molecular Cloning and Analysis of Candidate Genetic Loci

Alcohol dehydrogenases

A full-length human Class III alcohol dehydrogenase coding sequence was cloned from a liver cDNA library using oligonucleotide probes corresponding to peptide sequence from liver Class III ADH purified by us. The DNA sequence is consistent with recently-reported protein Class III ADH sequence and encodes 373 amino acids. Availability of this probe has provided additional information as to the structure and expression of class III ADH. Three electrophoretic isoforms of Class III ADH are detectable by immobiline isoelectric focusing[7], however, a single mRNA size (1750 bases) is detected by the cDNA probe in a wide variety of tissues, including brain and fetal tissues. Class III ADH is highly distinctive at the protein level, showing identity to Class I beta ADH at 73.4% of amino acid residues and 64.7% of DNA coding base pairs, but for the third nucleotide in the codons the homology was only 39.4%. This indicates that the divergence of Class III ADH occurred remotely in time and underlines the point that Class III ADH is not isozymic with the other ADHs but is a highly distinct enzyme. A SacI RFLP has been detected which will be highly useful for linkage analysis.

A close relationship of the class I ADH genes on human chromosome 4 was demonstrated using genetic linkage and pulsed field gradient electrophoresis of large DNA fragments.[8] Our results extend previous work to indicate the existence of an ADH gene complex on chromosome 4; the three Class I genes reside on a DNA fragment of less than 1 million base pairs.

At the protein level, we have definitively shown that Class III ADH is the only alcohol dehydrogenase expressed to any significant extent in brain.[10] A polyclonal antibody was used to immunohistochemically map the distribution of Class III ADH in macaque brain. Class III ADH was shown to be widely distributed in brain, to be most highly concentrated in the subependymal layer and not in cerebellar Purkinje cells as had been reported. Extensive testing of substrates revealed that long chain fatty alcohols and omega-hydroxy fatty acids were the best substrates for this enzyme and that the identity of class III ADH may in fact be omega hydroxy fatty acid dehydrogenase. Any class I (low ethanol km) ADH was concentrated at least 200-fold by purification with an immobilized 4-methyl pyrazole (CAP-GAPP), affinity column. Nevertheless, class I ADH was undetectable, setting a very low upper limit on the amount of this enzyme which could be present in brain and available for the generation of acetaldehyde.

Tryptophan hydroxylase

Tryptophan hydroxylase is a member of the family of aromatic amino acid monooxygenases which also includes tyrosine hydroxylase and phenylalanine hydroxylase. Tryptophan hydroxylase is rate-limiting for the synthesis of serotonin. Low central turnover of serotonin has been strongly implicated in impulsive and aggressive behaviors, including suicidal and antisocial behaviors in humans, and has also been tied to ethanol preference in rodents. As a start to relate differences in impulsivity to events at the genetic level including genetic differences in this enzyme, we cloned a full length mouse tryptophan hydroxylase cDNA from a mastocytoma cDNA library. The sequence encodes 447 amino acids, 3 more than the rabbit pineal tryptophan hydroxylase. The 3 extra amino acid residue are the result of a small duplication beginning at amino acid residue 6 and yielding the sequence KENKEN (k=lysine, E=glutamine, N=asparagine). This mouse tryptophan hydroxylase shows a high degree of homology with rabbit pineal tryptophan hydroxylase (87.6% of amino acid residues are identical) and considerable (approximately 50%) homology with other aromatic amino acid monooxygenases such as tyrosine hydroxylase and phenylalanine hydroxylase. Two sizes of messenger RNA are recognized by this probe in various mastocytoma cell lines and also in pineal gland. One is 1750 bases in size and the other is 4000 bases. However, midbrain tryptophan hydroxylase mRNA is not detectably hybridize to the mastocytoma tryptophan hydroxylase cDNA, indicating that the brain and peripheral (mastocytoma/pineal) enzymes are distinct.

Significance to Biomedical Research and the Program of the Institute:

Linkage studies in mice, discovery of a possible locus for control of ethanol intake

If confirmed by additional genetic studies, the LTW-4 ethanol intake locus on mouse chromosome 1 will provide an additional probe for detecting human genetic loci determining differences in the vulnerability to alcoholism. The availability of the mouse genetic model will be highly advantageous for determining whether this is the actual locus or one that is closely linked genetically, for studying the mechanism of action and for exploring methods of modifying ethanol preference.

Linkage studies in humans

Familial linkage studies offer a direct method of discovering new genetic determinants and markers for vulnerability to alcoholism, bridging the gap between final behavioral phenotypes, including alcoholism, and the fundamental molecular mechanisms. They also offer a definitive method for testing specific hypotheses regarding the effects of variants at candidate genetic loci including neurotransmitter biosynthesis, receptor and alcohol metabolic loci. Our establishment of methods for clinical phenotyping of families, primary culture of lymphoblastoid cell lines and large scale DNA extraction and typing of 150 protein and DNA polymorphisms have carried us much closer to the goal of compleing linkage studies on a significant group of families with alcoholism.

Candidate Locus Studies on Alcohol Dehydrogenases

The alcohol dehydrogenases are the primary enzymes responsible for the metabolism of ethanol. With our cloning of the class III alcohol dehydrogenase cDNA, DNA probes for all of the known human alcohol dehydrogenases are now available. This will allow new advances to be made in the understanding in the structural organization, functional enzymology and control of expression of these genes. For example, our results with the Class III ADH probe have already revealed that this gene is contitutively transcribed in most or all tissues and in fetal life. Also, class III ADH is structurally highly distinct and divergent from the other ADH's. These facts will make Class III ADH particularly interesting for structural genetic studies and studies of control of ADH expression. Our demonstration that the Class I alcohol dehydrogenases reside on a single DNA segment of less than 1 million base pairs indicates that they exist as a gene complex and raises the possibility that Class II and Class III ADHs, which also map to chromosome 4, may be part of the same complex. The positioning of the ADHs in a gene complex has important implications for their regulation, evolution and genetic variation.

Candidate Locus Studies on Tryptophan Hydroxylase

As mentioned above, tryptophan hydroxylase is rate-limiting for the synthesis of serotonin and diminished turnover of this neurotransmitter results in impulsive and aggressive behaviors. Such behaviors are prominent features of so called Type II alcoholism, a highly genetically penetrant, largely male limited form of alcoholism marked by early onset and often accompanied by antisocial behaviors. Our cloning of the mouse mastocytoma tryptophan hydroxylase cDNA provides a genetic probe which may be useful for obtaining the tryptophan hydroxylase gene expressed in midbrain and for improving at a fundamental level our understanding of the structure, function and expression of these important enzymes.

Proposed Course:

For human linkage studies, collection of families and typing of DNA and protein polymorphisms as linkage markers will continue as before except that a large number of families will be collected this year and additional neuropsychological phenotypes will be obtained. We will attempt to confirm the mapping of the possible locus for ethanol intake in the mouse.

For candidate locus studies on alcohol dehydrogenases, we will extend our studies on the ADH gene complex, testing whether Class III ADH is part of this complex and attempting to establish gene order.

For candidate locus studies on tryptophan hydroxylase, we will attempt to isolate a brain tryptophan hydroxylase cDNA and then identify individuals and mouse strains with functional genetic variants of this enzyme.

Publications:

Goldman D, Pikus HJ. Genetically variant, chromosomally mapped, and identified mouse brain proteins for behavioral analysis, Proc of the IVth World Congress of Biol Psychiat 1986;1262-1264.

Goldman D, Giri PR, O'Brien SJ. A molecular phylogeny of the hominoid primates as indicated by two-dimensional protein electrophoresis, Proc Natl Acad Sci USA 84, 1987;3307-3311.

Goldman D. Molecular markers for linkage of genetic loci contributing to alcoholism. Recent Developments in Alcoholism, Vol VI. In: Galanter M, ed. Recent Developments in Alcoholism, Vol VI. New York: Plenum Press, 1988; 333-349.

Goldman D, Lister RG, Crabbe JC. Mapping of a putative genetic locus determining ethanol intake in the mouse, Brain Res 1987;420:220-6.

Moss HB, Salin-Pascual RJ, Giri PR, Goldman D, Tamarkin L. Sex-differences in ethanol sensitivity and alcohol and aldehyde dehydrogenase activities in the Syrian hamster, Alcoholism 1987;7:301-7.

Goldman D, Merril CR. Protein polymorphisms detected by two-dimensional electrophoresis: an analysis of overall informativeness of a panel of linkage markers, J Psychiat Res 1987;21:597-608.

Valkonen KH, Goldman D. Purification and characterization of three forms of class III alcohol dehydrogenase, Electrophoresis 1988;9:132-5.

Goldman D, Cotton RW. Review of the molecular biology of the human alcohol dehydrogenase genes and gene products, Adv Alco Subst Abuse, in press.

Goldman D, Giri PR, O'Brien SJ. The phylogeny of the bears, giant panda and procyonids as indicated by two and one dimensional electrophoresis, Evolution, in press.

Giri PR, Linnoila, M, O'Neill JB, Goldman D. Identity of human brain alcohol dehydrogenase as a class III enzyme and its probable metabolic role, Brain Res, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00239-05 LCS

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.)
Alcoholism-Associated Cognitive Impairment and Organic Brain Syndrome

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

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	R. Rawlings	Mathematical Statistician	DBE, NIAAA

COOPERATING UNITS (if any)
United States Soldiers' and Airmen's Home, Washington, D.C.(N. Keller, A. Law, G. Smith); GWU (H. Weingartner)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Brain Research

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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

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

The purpose of this study is to examine the neuropsychological performance of several clinically defined populations of detoxified male alcoholics. Comparisons will be made among detoxified alcoholics with clinically defined chronic organic brain syndromes, dementia or amnestic syndrome; less cognitively impaired alcoholics who are in alcoholism treatment programs; and nonalcoholic controls.

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

PROJECT DESCRIPTION:

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
R. Rawlings	Mathematical Statistician	DBE, NIAAA
N. Keller	Staff Psychiatrist	MHS, USSH
A. Law	Chief	MHS, USSH
G. Smith	Chief	MHS, USSH
H. Weingartner	Professor	George Washington University

Objectives:

Chronic alcohol abuse may lead to two clinically and neuropathologically distinguishable syndromes: alcoholic dementia and alcoholic amnestic syndrome (called Korsakoff's psychosis), which together constitute the second most common cause of dementia in adults (approximately 10%). These two alcohol-related organic brain syndromes may represent the extremes on a cognitive dysfunction scale with alcoholic dementia characterized by a global intellectual decline, whereas alcoholic amnestic syndrome can be characterized as a severe and persistent amnesia with a relative sparing of other intellectual functions. The majority of alcoholic patients in clinical practice fall somewhere in between. In the present study, we propose to use a comprehensive battery of neuropsychological tests to differentiate alcoholic dementia from alcoholic amnestic syndromes. Less cognitively impaired alcoholics and normal, age-matched volunteers will be evaluated similarly. Comparisons among these groups will lead to a better characterization of cognitive similarities and differences in these groups.

Methods Employed:

Two clinically defined groups of alcoholics will be evaluated: participants in an alcoholism treatment program and those with sufficient, clinically defined, cognitive impairment so as to be judged not likely to benefit from the treatment program. The latter group will be separated by neuropsychological performance into those with alcoholic dementia and those with alcoholic amnestic syndrome.

It has been shown previously that the neuropsychological performance of neurologically impaired alcholics with dementia can be differentiated from that of alcoholics with amnestic syndrome. We plan to use a more comprehensive and sensitive test battery to better understand this differentiation.

Initially, each subject is participating in four sessions on four separate days. The first three involve a detailed neuropsychological assessment of intelligence, memory, and other cognitive functions. The fourth involves the collection of socioeconomic information, personality assessment, childhood history of hyperactivity, and drug use history, including alcohol.

The neuropsychological test battery is designed to obtain a global assessment of cognitive skills, an in-depth examination of memory functions, and an assessment of alcoholism-related cognitive decrements. The examination will take about 12 hours to complete. The battery consists of Halstead-Reitan Battery including Trails A and B; Wechsler Adult Intelligence Scale; Wechsler Memory Scale; Wisconsin Card Sorting Test; and selected memory tests designed to compare episodic versus semantic learning, automatic versus effortful learning, and language versus nonlanguage learning.

Scales designed to evaluate MBD and hyperactivity during childhood will also be administered.

Major Findings:

We are still collecting data and have begun to analyze it.

Significance to Biomedical Research and the Program of the Institute:

It has been well documented that alcoholics have impaired brain function. The present study is designed to better characterize the cognitive deficits observed in alcoholics so that more appropriate pharmacological intervention will be possible.

Proposed Course:

Data collection and analysis are continuing.

Publications:

Eckardt MJ, Martin PR. Diagnosis and treatment of chronic organic brain syndromes associated with alcoholism, Substance Abuse 1988;8:14-26.

Martin PR, Mukherjee AB, Eckardt MJ. Alcoholic organic brain disease, Proc IVth World Congress of Biological Psychiatry. New York: Elsevier, 1986; 1220-2.

Linnoila M, Eckardt MJ, Durcan M, Lister R, Martin PR. Interaction of serotonin with ethanol: clinical and animal studies, Psychopharm Bull 1987;23:452-7.

Martin PR, Eckardt MJ, Linnoila M. Treatment of chronic organic mental disorders associated with alcoholism. Galanter M, ed. Recent Developments in Alcoholism, Vol 7, New York: Plenum Press (in press).

Stapleton JM, Eckardt MJ, Martin PR, Adinoff B, Varner JL, Lane EA, Eckardt MJ, Linnoila M. Treatment of alcoholic organic brain syndrome with the serotonin reuptake inhibitor fluvoxamine: A preliminary study. Adv Alc Subst Abuse (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00240-09 LCS

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.)
Cognitive Function in Male Alcoholics

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

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Others:	R. Rawlings	Mathematical Statistician	DBE, NIAAA

COOPERATING UNITS (if any)
Department of Psychiatry and Human Behavior, University of California, Irvine (L. Gottschalk)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Brain Research

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

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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.)

This series of studies is concerned with cognitive function in detoxified male alcoholics. Recent and chronic alcohol consumption variables were found to interact with each other and with age and education in a non-linear fashion in predicting neuropsychological performance. Increased consumption predicted decreased performance even on tests whose mean scores were in the normal range. Little or no improvement in performance was demonstrable with short-term abstinence (14 - 20 days), although long-term abstinence (7 months) was associated with improvement. Similarly, hepatic and hematologic characteristics of long-term abstainers improved, whereas these systems functioned abnormally in people who continued to consume alcoholic beverages, albeit at significantly reduced levels. Relationships between various pretreatment prediction variables and subsequent outcome are also being studied. Increased risk of relapse was associated with excessive drinkers who were relatively early in their alcoholic careers as assessed by years of abusive drinking and accumulated lifetime exposure to alcohol. Although statistically significant relationships were observed between scores on certain neuropsychological tests and posttreatment alcohol consumption, neurospychological evaluation was determined to be of limited clinical utility.

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

PROJECT DESCRIPTION:

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
R. Rawlings	Mathematical Statistician	DBE, NIAAA
L. Gottschalk	Professor	Univ. of CA, Irvine

Objectives:

The present series of studies was designed to document the presence of CNS impairment in male alcoholics, discern possible etiological factors related to this impairment, and determine whether improvement in function is associated with subsequent abstinence. Additional questions concern whether treatment should commence immediately after detoxification, relationships between CNS function and treatment outcome, and neuropsychological consequences of post-treatment alcohol consumption.

Methods Employed:

A battery of 24 neuropsychological tests was administered to drug-free alcoholic inpatients (n=91) within 7 days of their last drink and again 17 days later. To control for practice effects, a nonalcoholic medical control group (n=20) also took the test battery twice, with approximately the same interval elapsing between administrations. Another group of alcoholic inpatients (n=32) took the tests only once, 14-31 days after their last drink. After patients completed the 21-day treatment program, they were contacted on a monthly basis to determine drinking behavior. At the end of 7 months, they returned to the hospital. Before testing, a breathalyzer and/or clinical laboratory determination of blood alcohol level was carried out in an attempt to ensure sobriety during testing. The 24 cognitive tests were then administered in a random order. Self administered questionnaires were used to calculate post-treatment frequency of drinking alcohol and quantity consumed per occasion. Patient-supplied collaterals were then contacted to verify the patients' self-reports. Approximately 24 months after entrance into the treatment program, 17 of the original 91 patients were located and agreed to take again the entire battery of neuropsychological tests.

Major Findings:

Cognitive performance in drug-free alcoholic men is significantly predicted by chronic and recent drinking practices. Furthermore, it appears that certain patterns of consumption may accelerate the alcohol-induced decline of brain function. Little or no improvement in cognitive performance was demonstrable with short-term abstinence, when controls were included for the effects of repeated testing. Continued alcohol consumption by recovering alcoholics is associated with reduced cognitive performance, while those who abstained have improved test scores. Neuropsychological performances determined 24 months after entrance into the program were at the same levels as at 7 months after entrance.

Similar findings were observed in clinical laboratory tests, with long-term abstainers (7 months) having improved hepatic and hematologic functioning in contrast to the continued abnormal functioning observed in those people who continued to drink, albeit at significantly reduced levels. Further analysis of these clinical laboratory tests revealed widespread and persistent alcoholism-related alterations in organ system functioning even after long-term abstinence (7 or 24 months).

Male alcoholics' pretreatment levels of alcohol consumption were found to be related statistically to posttreatment levels of consumption with an increased risk of relapse associated with excessive drinkers who were relatively early in their alcoholic careers as assessed by years of abusive drinking and accumulated lifetime exposure to alcohol.

Statistically significant relationships were observed between neuropsychological test scores and posttreatment alcohol consumption determined eight months after completing treatment for 72 alcoholics. These results, however, were influenced by the particular measure of posttreatment consumption evaluated, type of statistical analysis, and whether the entire sample of 72 or a subsample of 55 with consistently reported drinking information was used. Scores of tests most consistently showing relationships were often counter to the notion that increased neuropsychological performance predicts more favorable treatment outcome. Discriminant analysis resulted in 70% correct classification, with chance being 50%. It is concluded that neuropsychological evaluation is of limited clinical utility in predicting posttreatment alcohol consumption.

Significance to Biomedical Research and the Program of the Institute:

Recent and chronic drinking practices appear to have adverse and possible direct effects on brain function in male alcoholics. Insofar as decisions about the initiation of therapeutic interventions which rely on cognitive processes are based on neuropsychological performance, we conclude that treatments may commence as soon as the clinical symptoms associated with acute withdrawal have subsided. Continued alcohol consumption by recovering alcoholics might serve to maintain cognitive performance at reduced levels, and this possibility should be considered in determining appropriate treatment goals for alcoholic patients. Neuropsychological evaluation is of limited clinical utility in predicting posttreatment alcohol consumption. However, it may be of value in assisting treatment staff in obtaining cognitively appropriate posttreatment employment for patients and in more effectively individualizing treatment, but this remains to be demonstrated.

Proposed Course:

Data analysis will be continued, and the results will be published in appropriate scientific journals.

Publications:

Eckardt MJ, Rawlings RR, Graubard BI, Faden V. Martin PR, Gottschalk LA. Neuropsychological performance and treatment outcome in male alcoholics, Alcoholism Clin Exp Res 1988;12:88-93.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00267-03 LCS

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.*)

Brain Imaging

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

	Name	Title	Affiliation
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Others:	M. Linnoila	Chief	LCS, NIAAA
	R. Rawlings	Mathematical Statistician	DBE, NIAAA
	D. Rio	Physicist	LCS, NIAAA
	J. Rohrbaugh	Research Psychologist	LCS, NIAAA
	J. Stapleton	Staff Fellow	LCS, NIAAA

COOPERATING UNITS (*if any*)

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) Minors
☐ (a2) Interviews

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

Various clinical imaging methods are being used to study the brain in vivo. These techniques enable comparisons of gross anatomy (CAT - Computed Axial Tomography; MRI - Magnetic Resonance Imaging) of the brain with electrical activity (EEG - electroencephalography; ERPs - Event-Related Potentials) and rate of glucose utilization in specific regions (PET - Positron Emission Tomography). From a clinical perspective, these techniques, in association with other diagnostic tests, enable qualitative judgments to be made as to the anatomic and physiologic integrity of the brain. In order to quantitatively analyze image data, the imaging techniques themselves are being investigated, as well as the effects of the associated mathematical models and subjective inputs on the reconstruction of the brain image. Moreover, mathematical and statistical methods for evaluating and relating these various sources of multivariate data are being developed.

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

PROJECT DESCRIPTION:

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
E. Lamoreaux	Computer Programmer	ROB, NCI
M. Linnoila	Chief	LCS, NIAAA
R. Rawlings	Mathematical Statistician	DBE, NIAAA
D. Rio	Physicist	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
J. Stapleton	Staff Fellow	LCS, NIAAA

Objectives:

Our goals are 1) to develop three-dimensional PET and CAT/MRI images which are superimposable, and 2) to develop new mathematical and statistical methods to assess these different types of multivariate image data and determine relationships between these images and brain electrical activity monitored at the scalp.

Methods Employed:

A critical review of the raw and reconstructed data obtained from PET, CAT and MRI is being carried out to insure that sources of noise inherent in each technique are taken into account thereby insuring that artifacts will be correctly eliminated and confidence intervals may be more accurately estimated, leaving only statistically significant differences. Emphasis is currently on self-attenuation within the brain-skull system, applicability of the glucose utilization model, and software filters used to reconstruct images.

Two of the main issues involved in this area are patient position monitoring during a scan and patient repositioning, either on the same scanner at different times or on another scanner. Currently, two methods of recording patient position are being tested. The first system consists of a magnetic transducer attached to the patient, with associated pickup attached to the various scanners which will enable us to determine the position of a "stable point" on the patient and a computer system which will display this information both numerically and graphically. The second system consists of a mechanical system designed to ride on the patient's face to provide more quantitative visual cues as to patient movement without the complications of the aforementioned system.

All data obtained from the scanners are processed and displayed on our own image processing system enabling us to analyze basic pixel data instead of post-processed images. Individual brain slices are oriented in three dimensions and displayed as stacked three dimensional data or as surface contours. Using these methods it will be possible to display and calculate regional volumes and superimpose data from CAT, PET and MRI as well as other sources. This will insure that a regional alteration in metabolism corresponds to a particular anatomical location.

Resulting data are analyzed with mathematical techniques used in image processing, pattern recognition, and spectral analysis, i.e., by representing the spatial data in frequency space. Statistical tests are then used to study differences between various clinical populations.

Procedures are being developed to calculate and display scalp-monitored electrical potential fields and to estimate the position and distribution of electrical sources in the brain producing these potentials. This will enable us to correlate anatomical or metabolic changes in the brain with modifications of cognitive processes as represented by changes in evoked responses.

Major Findings:

Data for this study are still being collected and analyzed.

Significance to Biomedical Research and the Program of the Institute:

Establishing structure and function relationships among various areas of the brain is a crucial step in determining mechanisms. The approach advocated in the research described herein emphasizes 1) detailed and intensive assessment of relatively few, carefully selected patients, thereby reducing heterogeneity in patient characteristics and enabling a convergence of information, and 2) comparing three-dimensional PET, CAT and MRI images with each other and with electrical sources derived from scalp-monitored EEG and ERPs. Such studies have yet to be reported in the literature. Successfully combining these techniques would be a significant accomplishment with obvious applicability to other studies of brain structure and function.

Proposed Course:

Data are being collected and analyzed. As analyses are completed, the results will be published in appropriate scientific journals.

Publications:

Eckardt MJ, Rohrbaugh JW, Rio D, Rawlings RR, Coppola R. Brain imaging in alcoholic patients, Adv Alc Subst Abuse, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00268-03 LCS

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.)

Behavioral Effects of Alcohol and Other Psychotropic Drugs

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

PI:	R. Lister	Visiting Associate	LCS, NIAAA
Others:	M. Durcan	Visiting Fellow	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	D. Goldman	Section Chief	LCS, NIAAA
	L. Hilakivi	Visiting Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	D. Nutt	Visiting Scientist	LCS, NIAAA

COOPERATING UNITS (if any)

George Washington University (H. Weingartner); VA Medical Center, Portland, OR (J. Crabbe); United States Soldiers' and Airmen's Home, Washington, DC

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Brain Research

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

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

To determine the psychobiological distinctiveness of various behavioral processes, pharmacological and genetic methods are being used. The relationships among alcohol's anxiolytic, reinforcing, amnestic, locomotor stimulant, sedative/hypnotic and hypothermic effects are being studied using a variety of inbred strains of mice. A new paradigm has been established to examine alcohol's effects on social and aggressive behavior. The interactions of ethanol with with various drugs that interact specifically with different neurotransmitter systems are also being investigated. A variety of benzodiazepine receptor inverse agonists have been found to partially reverse some of the behavioral effects of ethanol. Specific alpha-2 adrenoceptor antagonists are also capable of reversing some of ethanol's effects.

A variety of different methods are being used to investigate mechanisms of learning and memory. The cognitive functioning both of normal human volunteers under the influence of different drugs (such as alcohol and benzodiazepines), and of various patient populations (e.g., subjects with Korsakoff's psychosis, various dementias) are being examined. Finally, the effect of changes in mood on cognitive functions is being examined in normal volunteers following treatment with alcohol.

PROJECT DESCRIPTION:

Investigators:

R. Lister	Visiting Associate	LCS, NIAAA
M. Durcan	Visiting Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
D. Goldman	Section Chief	LCS, NIAAA
L. Hilakivi	Visiting Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Nutt	Visiting Scientist	LCS, NIAAA
H. Weingartner	Professor	George Wash. Univ.
J. Crabbe	Staff Scientist	VA Med. Ctr., Portland, OR

Objectives:

The behavioral effects of alcohol in rodents have been most extensively investigated using tests sensitive to the drug's hypnotic, hypothermic, psychomotor impairing, and sedative properties. In contrast, ethanol's anxiolytic, anticonvulsant, amnestic and reinforcing properties have been studied less thoroughly, largely because tests for these effects are more difficult to develop. We are currently developing tests suitable for investigating these latter properties of ethanol in mice. Following validation of the tests, we will investigate relationships between alcohol's various behavioral effects using a large number of inbred strains of mice which differ in their behavioral responses to alcohol. The results of these experiments should allow us to determine which behavioral effects of ethanol are related. Behavioral genetic techniques will then be applied to investigate the genetic basis for the differences in response to alcohol.

Alcohol shares many properties with drugs acting at the benzodiazepine-GABA receptor macromolecular complex. In order to determine which (if any) of its effects are mediated through this site, the interactions of alcohol with various ligands for the complex are being investigated in the behavioral tests.

It has been suggested that alcoholics can be divided into at least two different groups, one of which is associated with impulsive and often aggressive behavior. This form of alcoholism has a high heritability. In order to better understand the genetic and neurochemical bases of this form of alcoholism we are developing animal models of aggressive and impulsive behavior. We are investigating aggressive behavior in mice in a variety of different paradigms, examining the effects of alcohol, and comparing the behavior of different strains of mice. Behavioral genetic methods will then be applied in an attempt to understand the mechanisms underlying strain differences.

Several lines of evidence suggest that learning and memory can be studied in a number of different forms. For example, a subject may appear to be amnesic when a test of episodic learning is used, but perform normally in a test of procedural learning. In the present series of studies we are attempting to determine which cognitive processes are distinct. We believe that cognitive dysfunction can yield information about normal cognitive func-

tioning. Therefore, we are comparing the performance of several populations of subjects in a battery of cognitive tests. The subjects we are testing include normal volunteers under the influence of either alcohol or a benzodiazepine and patients with various memory disorders including Korsakoff's disease, Alzheimer's disease and alcoholic dementia. By determining what these populations can and cannot learn and remember, we expect to provide evidence to support distinctions between various cognitive processes.

We are also investigating the effect of drug-induced alterations in mood on cognitive function. It is likely that ethanol is self-administered at least in part for its mood-elevating effects. Very little is known in this area, however, and what has been reported is generally anecdotal rather than experimental. We are examining changes in mood following the consumption of alcohol and examining how such changes alter several cognitive processes.

Methods Employed:

The plus-maze test of anxiety is used to assess anxiolytic effects of various drugs. Mice are placed on an elevated plus-maze consisting of two open and two closed arms. The proportion of time spent on the open arms expressed as a percentage of the total time on both types of arms, and the percentage of arm entries made into the open arms are used as two indices of anxiety. Animals are tested for 5 min in a holeboard apparatus immediately before the test to give measures of locomotion and directed exploration.

A test of social and aggressive behavior has been developed that involves placing pairs of male mice in an unfamiliar arena, and scoring their behavior for a period of 5 min. The mice are then returned to their home cages. The following day each mouse is individually familiarized with the test arena. The day after this, both mice are again placed in the test arena and their social behavior scored.

The data gathered from these tests will be added to data already collected in the same strains by Dr. John Crabbe (VA, Portland, OR) on other behavioral effects of alcohol (hypnotic, hypothermic, and ataxic). It should be possible to determine which of ethanol's effects are related. A genetic analysis aimed at determining the number of loci responsible for strain differences is being performed by Dr. David Goldman.

To assess the interactions of ethanol with various drugs that interact with different neurotransmitter systems, six different paradigms are being used: the holeboard; the plus-maze; the test of social behavior; an observer-rated test of intoxication; a measure of duration of loss of righting reflex; and a seizure threshold paradigm in which seizure threshold to the convulsant bicuculline is determined.

An extensive test of learning and memory has been developed for use in humans which assesses: episodic memory, procedural learning, several different forms

of priming, retrieval of information from semantic memory, and recognition memory. This test is being given to patients with Korsakoff's psychosis, Alzheimer's disease or alcoholic dementia. A shorter version is being used to investigate the acute effects of ethanol on these various memory functions.

In experiments investigating interactions between mood and cognition, normal volunteers are being studied. They are given a variety of tests to perform, and mood-ratings questionnaires to complete, in both a sober state and following the consumption of an alcohol-containing beverage, on the rising portion of the blood-alcohol curve. The battery of cognitive tests has been designed to assess the effects of mood on the encoding and retrieval of information of different affective tone. Both semantic and episodic memory function are assessed.

Major Findings:

The benzodiazepine receptor ligand Ro 15-4513 has been found to partially reverse the reduction in exploration caused by ethanol in the holeboard test, the reduction in anxiety caused by ethanol in the plus-maze test, the reduction in social behavior caused by ethanol, and the anticonvulsant effects of ethanol in the seizure threshold paradigm. In all these tests another benzodiazepine receptor partial inverse agonist (FG 7142) produces similar effects. Ro 15-3505, a structural analogue of Ro 15-4513, was also found to be able to partially reverse ethanol's effects in these tests and to possess less marked inverse agonist activity (it reversed some of the inverse agonist effects of Ro 15-4513). In the intoxication paradigm Ro 15-4513, unlike FG 7142, was able to reduce the intoxicating effect of ethanol. Further, FG 7142 reversed the antagonism of ethanol's effects by Ro 15-4513.

The alpha-2 adrenoceptor antagonists atipamezole and idazoxan were both capable of reducing ethanol's effects on exploration in the holeboard test. They also reduced ethanol's effects in the observer-rated test of intoxication. In contrast to the benzodiazepine receptor partial inverse agonists, neither atipamezole nor idazoxan were anxiogenic in the plus-maze or proconvulsant in the test of seizure threshold.

The 5-HT reuptake inhibitor fluoxetine, selectively reduced ethanol's anxiolytic effect on the plus-maze, failing to alter ethanol's effects in the holeboard test. Other serotonin reuptake inhibitors, however, failed to produce a similar effect. The data suggest that the ability of serotonin reuptake inhibitors as a class to reduce ethanol consumption does not result from a modification of ethanol's anxiolytic or stimulant properties.

In the test of social behavior, increasing periods of social isolation increased both social and aggressive behavior. Familiarity with the test arena and low levels of illumination also increased social behavior. Ethanol exerted a biphasic effect on aggressive behavior, low doses increasing and high doses decreasing the incidence of aggression.

Significance to Biomedical Research and the Program of the Institute:

Alcoholism has a significant genetic component. It is important, therefore, to develop animal models that not only measure processes that contribute to the development of alcohol dependence, but that are also amenable to genetic analysis. Cloninger has proposed that alcoholics can be divided into two types. Type 1 alcoholics have been classified as anxious. They often drink to relieve anxiety. Type 2 alcoholics are impulsive and often aggressive. We are developing and using animal models of anxiety and impulsive/aggressive behavior in an attempt to understand the genetic and environmental variables that may be important in the different forms of human alcoholism.

The neurobiological mechanisms underlying ethanol's many behavioral effects are poorly understood. We believe that we may gain insight into these mechanisms by examining the interactions of ethanol with drugs with highly specific and well-characterized mechanisms of action. Our understanding of these mechanisms will be invaluable in the development of drugs that might reduce voluntary ethanol consumption, and drugs that might antagonize the potentially lethal effects of high doses of ethanol.

The ability to distinguish between various basic processes involved in learning and memory is of fundamental importance both in the diagnosis and treatment of memory disorders. We hope to identify analogous processes in humans and laboratory animals using the present approach, and this will allow a more efficient screening of potential treatments.

Previous studies have shown that mood can have quite marked effects on the way information is retrieved and acquired. However, the methods used to induce different moods in these studies have been subject to criticism. The current studies circumvent a number of these problems by using drugs as the means of altering mood. If we are able to demonstrate that ethanol alters cognitive processing via its effects on mood, this may not only add to our understanding of why people drink, but will also provide cognitive psychologists with a valuable method to further investigate how mood can affect cognition.

Proposed Course:

We shall continue gathering data using the tests outlined above in several inbred strains of mice. The interactions of ethanol with drugs acting on various neurotransmitter systems will be investigated and compared with data already obtained. Environmental and genetic influences on aggressive behavior will be related to differences in serotonergic function.

Publications:

Nutt DJ, and Lister RG. The effect of the imidazodiazepine Ro 15-4513 on the anticonvulsant effects of diazepam, sodium pentobarbital and ethanol, Brain Res 1987;413:193-196.

Lister RG. Interactions of Ro 15-4513 with diazepam, sodium pentobarbital and ethanol in a holeboard test, Pharmacol Biochem Behav 1987;28:75-79.

Lister RG. Reversal of the intrinsic effects of Ro 15-4513 on exploratory behavior by two benzodiazepine receptor antagonists, Neurosci Lett 1987;79: 306-310.

Lister RG. The benzodiazepine receptor inverse agonists FG 7142 and Ro 15-4513 both reverse some of the behavioral effects of ethanol in a holeboard test, Life Sci 1987;41:1481-1489.

Lister RG, Karanian J. Ro 15-4513 induces seizures in DBA/2 mice undergoing ethanol withdrawal, Alcohol 1987;4:409-411.

Lister RG, Nutt DJ. Interactions of the imidazodiazepine Ro 15-4513 with chemical convulsants, Br J Pharmacol 1988;93:210-214.

Nutt DJ, Lister RG. Strain differences in response to a benzodiazepine receptor inverse agonist (FG 7142) in mice, Psychopharmacology 1988;94:435-436.

Lister RG. Behavioral interactions between ethanol and imidazodiazepines with high affinities for benzodiazepine receptors, Life Sci 1988;42:1385-1393.

Lister RG. Antagonism of the behavioral effects of ethanol, sodium pento-barbital and Ro 15-4513 by the imidazodiazepine Ro 15-3505, Neurosci Res Commun 1988;2:85-92.

Durcan MJ, Lister RG. Time course of ethanol's effects on locomotor activity, exploration and anxiety in mice, Psychopharmacology, in press.

Durcan MJ, Lister RG, Eckardt MJ, Linnoila M. Interactions of 5HT reuptake inhibitors and ethanol in tests of exploration and anxiety, Adv Alcohol Subst Abuse, in press .

Lister RG. Interactions of three benzodiazepine receptor partial inverse agonists with ethanol in a plus-maze test of anxiety, Pharmacol Biochem Behav in press.

Nutt DJ, Lister RG, Rusche D, Bonetti EP, Reese RE, Rufener R. Ro 15-4513 does not protect rats against the lethal effect of ethanol, Eur J Pharmacol in press.

Durcan MJ, Lister RG, Eckardt MJ, Linnoila M. Behavioral interactions of fluoxetine and other 5-hydroxytryptamine uptake inhibitors with ethanol in tests of anxiety, locomotion and exploration, Psychopharmacology, in press.

Lister RG, Hilakivi LA. The effects of novelty, isolation, light and ethanol on the social behavior of mice, Psychopharmacology, in press.

Lister RG. Reversal of ethanol-induced reductions in exploration by two benzodiazepine antagonists (flumazenil and ZK 93426), Brain Res Bull, in press.

Nutt DJ, Lister RG. Antagonising the anticonvulsant effect of ethanol using drugs acting at the benzodiazepine/GABA receptor complex, Pharmacol Biochem Behav, in press.

Lister RG. Interactions of ethanol with benzodiazepine receptor ligands in tests of exploration, locomotion and anxiety. Pharmacol Biochem Behav, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00250-05 LCS

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.)*

Electrophysiological Studies of Acute and Chronic Alcohol Consumption

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

PI:	J. Rohrbaugh	Research Psychologist	LCS, NIAAA
Others:	B. Adinoff	Senior Staff Fellow	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	D. Rio	Physicist	LCS, NIAAA
	J. Stapleton	Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*

Department of Psychology, Catholic University (R. Parasuraman); Department of Electrical Engineering, University of Nebraska (J. Varner)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Brain Research

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Although alcohol clearly leads to alterations in mental processes and behavior, it is perhaps because these effects are so great and so pervasive that they have proven difficult to characterize. The global performance measures commonly used to document such effects, while demonstrating clearly their presence, often provide little information as to the specific locus or mechanism of effect. The present research aims to parse the separate effects of alcohol on peripheral nerve, sensory, cognitive and motor systems using event related brain electrical potentials. These potentials are studied within a broad context provided by performance, psychophysiological, neuropsychological, neuroradiological and neuropsychiatric data.

The effects of alcohol are investigated in three classes of subjects: 1) The effects of acute administration are examined in normal volunteers with respect to dose-response relationships, the temporal course of effect, and relationship to blood alcohol levels; 2) Brain electrical activity is studied, in conjunction with neuropsychological data, in social drinkers in an attempt to assay the effects of moderate levels of alcohol consumption; 3) Abstaining alcoholics, including those with alcoholism-related mental impairment, are studied to characterize the deficits, to examine the short- and long-term recovery effects, if any, during abstinence, and to examine the efficacy of various treatment strategies.

PROJECT DESCRIPTION:

Investigators:

J. Rohrbaugh	Research Psychologist	LCS, NIAAA
B. Adinoff	Senior Staff Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Rio	Physicist	LCS, NIAAA
J. Stapleton	Staff Fellow	LCS, NIAAA
J. Varner	Associate Professor	Univ. of Nebraska
R. Parasuraman	Associate Professor	Catholic Univ.

Objectives:

This research aims to provide a comprehensive overview of the effects of alcohol, both acute and chronic, on sensory, cognitive and motor systems. A primary focus is upon event-related brain electrical potentials, elicited in response to environmental stimulation, and extracted by computer from the on-going EEG. The electrical responses will be studied in various tasks and under various conditions for information about the related neural processes and possible disturbances associated with alcohol consumption. Measurement of the brain electrical potentials will be accompanied by simultaneous measurement of psychophysical judgments, reaction time and other behavioral responses, as well as responses in autonomic and somatic systems (EKG, electrodermal, electromyographic, pupillary, vasomotor, respiratory and oculomotor responses).

Methods Employed:

Brain electrical activity and psychophysiological responses are measured from surface electrodes using conventional EEG and polygraph instruments, allowing data to be acquired simultaneously from as many as 40 channels. The responses are analyzed with respect to waveform and sensitivity to experimental variables using multivariate techniques. Topographic distributions of the responses over the scalp are studied for evidence of neural sources of the electrical activity, using scalp mapping and dipole inference techniques.

Sensory functions are evaluated separately for visual, acoustic and somatosensory systems using clinically validated techniques. Visual stimulation and recording techniques permit evaluation of function in retinal, optic nerve and tract, and cortical centers. Similarly, auditory and somatosensory techniques permit examination of function in peripheral, brainstem and cortical areas. Cognitive function is assessed by examination of responses that are related to attention and decision making as described representatively below:

1) Vigilance and habituation. Habituation of response amplitude or frequency upon repetitive stimulation is a primitive and ubiquitous form of behavioral plasticity that has been proposed to underlie a variety of complex learning and performance abilities. Data indicate that alcohol affects this process, as measured by a number of ERP, autonomic and behavioral measures. The decline in

responsiveness is particularly evident in sustained attention, or vigilance, situations. We are further investigating these changes in a number of paradigms, including traditional habituation, dishabituation and spontaneous recovery paradigms in which autonomic and ERP measures are studied. Performance measures are derived from a vigilance task that has been developed to show a particularly rapid rate of performance decrement, and thus allow the acute effects of alcohol to be studied separately at ascending and descending limbs of the blood alcohol curve. This vigilance task also permits separate examination of sensitivity and report criterion changes during the time of task.

2) Orienting. The orienting response is generally conceived as a transient attentional response to novel or significant stimuli. Although the response habituates rapidly to innocuous stimuli, some data indicate that the response to task-relevant stimuli may persist so long as salience is maintained. The response is believed to be generally important in a variety of memory, conditioning and cognitive skills. The effects of alcohol on orienting, are particularly conspicuous on a slow, late component of the ERP (the O wave), which we believe to be a central manifestation of orienting. The evidence for this belief includes observations that the eliciting conditions are appropriate, i.e. the O wave appears only in response to stimuli that are greatly discrepant from expected stimuli, or that are task relevant and significant. The wave is accompanied by autonomic responses that have more traditionally served to earmark orienting responses, and its temporal course is similar to that shown by performance measures of orienting. Moreover, it appears to originate in a right dorsolateral frontal area that is believed to be particularly important in the regulation of attention. The right frontal origins of the O wave suggest to us that its study is likely to be especially fruitful in light of hypotheses that the effects of alcohol selectively target functions subserved by frontal and/or right hemisphere cortices.

3) Effects of Attention on Sensory Systems. A long-favored strategy for studying attention is to examine responses to probe stimuli introduced during behaviorally different periods. We have developed an elaboration of this strategy that allows the extent, distribution across sensory modalities or channels, and temporal course of attentional allocation to be traced continuously. The probe stimuli in this technique are weak, background stimuli that are repetitively presented at a steady rate. Under such conditions, a steady EEG rhythm is established, which is believed to be composed of early responses in primary sensory cortices. Our technique is to establish this rhythm under conditions demanding various forms of attention, and to extract the rhythm from the composite EEG using digital filtering techniques for continuous examination of phase and amplitude. Studies from normal subjects indicate that the nature of this rhythm varies in a systematic and theoretically consistent manner; current studies are using this response to infer the appropriateness and extent of attention as influenced by the acute and chronic effects of alcohol.

4) Information Processing Stages. A powerful method, we believe, for identifying specific loci of alcohol's effects upon performance is presented by the additive factor method, in which information processing is presumed to involve a series of sequential stages. By manipulation of such features as stimulus in-

tensity, clarity or complexity, and assessing the effects on speeded response performance, the stages distrubed by alcohol can be infered. Examination of ERP components within this context provides convergent evidence. Particularly relevant is the "P300" component, which is a positive component peaking at latencies of 300 msec or greater post stimulus. Previous experiments have suggested that P300 is important because it is diminished in chronic alcoholics and in their offspring.

Major Findings:

A substantial portion of the year's effort has been devoted to the analysis of data obtained from patients diagnosed as suffering alcohol-related organic brain syndromes, primarily Korsakoff's disease. Data were obtained from fourteen patients who were studied intensively under baseline conditions and within context of an ongoing investigation of the effects associated with chronic administration of the serotonin reuptake inhibitor fluvoxamine. For comparison purposes, data were obtained from ten age-matched normal subjects as well. Preliminary analyses disclose a number of significant effects associated with diagnostic category and with chronic fluvoxamine administration. Electrophysiological tests of transmission time through the afferent brainstem auditory system, and anterior visual pathways, show increase$ conduction latencies in the alcoholic patients, in comparison to norm!l controls. These functional deficits may reflect a process of demylination associated with alcoholic organic brain disease, although the demylination may be a specific rather than general process as indicated by the fact that those subjects showing increased conduction times in the visual system were not necessarily those showing increased auditory conduction times. Examination of P300 and other long-latency ERP measures disclosed abnormalities in these components, and by inference the associated cognitive processes, as well. The P300s from alcoholic dementia patients were profoundly different than those from Korsakoff patients or normal subjects, showing reduced amplitude and sensitivity to task variables, and an apparent origin in frontal regions of the brain in contrast to the central-parietal focus usually obtained. The P300's from Korsakoff patients were also abnormal, although the abnormalities were more subtle and were not apparent in all experimental tasks. Rather, the abnormalities were most pronounced in a task wherein P300 was analyzed as a function of a sequential order in which stimuli were delivered to the patients. Whereas the P300's from normals gave evidence to a process of frequency monitoring and associated development of sequential expectancies, this process was much less evident in Korsakoff patients. These data may be interpreted as evidence for a deficit in the encoding of event probabilities, perhaps deriving from a defect in working memory for the immediately preceding context.

It was observed in addition that the EEG from the alcoholic patients differed markedly from that obtained in normals, as disclosed in spectral analyses and topographic mapping. A principal difference was the raw amplitude, which was much greater in the alcoholic patients at all frequencies and at all electrode sites. This difference was largely normalized by fluvoxamine. The EEGs from alcoholic patients were found also to show other fundamental differences in spectral composition.

Data collection has proceeded on a major topographic distribution study which will investigate the relationship of the steady EEG rhythm we have been studying to midlatency sensory responses as well as later components (O wave and P300) by comparing topographic characteristics in the same subjects. Data will also be used to further develop strategies for equivalent dipole modelling in order to explore commonalities in underlying electromotive generators.

Significance to Biomedical Research and the Program of the Institute:

These studies offer the prospect of characterizing more completely the extent and nature of the effects of alcohol on the nervous system. This information will be valuable in describing and accounting for the detrimental effects of alcohol on performance, and will in addition be of diagnostic and prognostic value in patients suffering adverse effects of chronic alcohol consumption.

Proposed Course:

Immediate plans are to complete analyses of the acute and chronic studies described above, and to complete data collection on the topographic mapping study. Continued emphasis will be placed on the development of paradigms and recording procedures that are selectively responsive to the effects of alcohol on various neural systems.

Publications:

Rohrbugh JW, Stapleton JM, Frowein HW, Adinoff B, Varner JL, Lane EA, Eckardt MJ, Linnoila M. Acute effects of ethanol on motor performance and movement-related brain potentials, Adv Alc Subst Abuse, in press.

Rohrbaugh JW, Stapleton JM, Parasuraman R, Zubovic E, Frowein HW, Adinoff B, Varner JL, Zubovic EA, Lane EA, Eckardt, MJ, Linnoila M. Alcohol intoxication reduces visual sustained attention, Psychopharmacology, in press.

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Rohrbaugh JW, Johnson R, Parasuraman R. eds. Event-Related Brain Potentials: Issues and Interdisciplinary Vantages. New York: Oxford University Press, in press.

Rohrbaugh JW, Chalupa LM, Lindsley DB. Evoked potentials related to decision confidence. Galbraith GC, Keitzman ML, Donchin E, eds. Neurophysiology and Psychophysiology: Experimental and Clinical Applications. Hillsdale, NJ, Erlbaum 1988;326-332.

Johnson R, Rohrbaugh J, Parasuraman R. eds. Current Trends in ERP Research, Amsterdam: Elsevier, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00237-06 LCS

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.)*
Individual Variability in Drug Metabolism by Carbon Dioxide Breath Tests

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

PI:	E. Lane	Senior Staff Fellow	LCS, NIAAA
Others:	M. Towle	Chemist	LCS, NIAAA
	B. Ravitz	Medical Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(If any)*
Cooperating Units: Epilepsy Branch, NINCDS (R. Porter); Nursing Dept., NINCDS (I. Naveau)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
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INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	0.8	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.)*
Preliminary testing of the prediction that caffeine, a low extraction ratio drug (0.1) should be a more sensitive probe of enzyme induction than methacetin, a high extraction ratio drug (0.9), when excretion of a metabolite (CO_2) is measured, has been carried out. A single dose of each was administered to 8 healthy volunteers and 9 epilepsy patients treated with phenytoin, carbamazepine and/or phenobarbital. The 13C carbon dioxide in expired breath was measured by isotope ratio mass spectrometry. The percentages of the dose excreted as CO_2 in 2 hr. were compared: 3.22% $\pm$ 0.86 and 5.54% $\pm$ 1.59 caffeine was excreted by controls and patients, respectively, compared with 28.6% $\pm$ 5.8 and 40.0% $\pm$ 4.2 methacetin. The results in the 2 subject groups were significantly different for both probes ($p<.05$). These data do not support the theoretical prediction that the extraction ratio of a drug has a critical effect upon its usefulness in detection of induction of oxidative metabolism via the carbon dioxide breath test. In addition, preliminary data illustrate the potential usefulness of this test to follow the time course of changes in drug metabolism/ liver function of an alcoholic patient when he stops drinking.

PROJECT DESCRIPTION:

Investigators:

E. Lane	Staff Fellow	LCS, NIAAA
M. Towle	Chemist	LCS, NIAAA
B. Ravitz	Medical Staff Fellow	LCS, NIAAA
I. Naveau	Clinical Nurse	Nursing Dept., NINCDS
R. Porter	Epilepsy Br	NINCDS

Objectives:

This project is designed to use a low risk and relatively noninvasive technique to evaluate the rate at which an individual patient can metabolize drugs.

Methods Employed:

The test drugs are labeled with a stable isotope of carbon (13-C) in a methyl group that is removed by liver enzymes. This labeled carbon then appears in expired carbon dioxide where it can be measured, using an isotope ratio mass spectrometer. Predictions of the suitability of particular drugs as probes for drug metabolizing ability are made using pharmacokinetic models and computer simulations. The test drug and the complimentary metabolite formed when the test drug is demethylated are measured in plasma by means of high performance liquid chromatography.

Major Findings:

The percentage of a dose of either a low (caffeine) or high (methacetin) extraction ratio drug excreted as CO_2 within 2 hr. of a dose was measured in a small sample of drug free healthy volunteers and epilpsy patients taking enzyme inducing anticonvulsants. The results were statistically significantly different for both probes, therefore, failing to support the theoretical prediction that the extraction ratio of a drug has a critical effect upon its usefulness in detection of induction of oxidative metabolism via measurement of excretion of the metabolite CO_2.

Significance to Biomedical Research and the Program of the Institute:

Many factors, including alcohol consumption, affect the ability of an individual to metabolize various drugs. A method, such as this breath test, for rapid evaluation of drug metabolizing ability, should improve individualization of drug treatments. This would have particular application to times when drug metabolizing ability is changing because of alcohol withdrawal and treatment of alcoholism.

Proposed Course:

Evaluation of this test in alcoholics, before and during treatment and in epileptic patients during treatment with enzyme inducing drugs, is proposed. Three drugs (aminopyrine, caffeine and methacetin) will be evaluated as probes in this liver function test.

Publications:

Lane EA. The aminopyrine breath test for the evaluation of liver function in alcoholic patients: Drug pharmacokinetics and environmental factors. Adv Alc Subst Abuse, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00248-05 LCS

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.)*
Acetylation Phenotype of Alcoholics

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

PI:	E. Lane	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Biochemistry and Pharmacology

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Any coincidence of the slow acetylator phenotype and alcoholism will be investigated in this study.

The proportion of alcoholic subjects that exhibits the slow acetylator phenotype has been compared with the proportion of normal volunteers that exhibits the slow acetylator phenotype.

The acetylator phenotype has been measured in normal volunteers and recovered alcoholics, who are not related to each other. The recovered alcoholics have a history of alcoholism and the normal volunteers are nonalcoholic and have no first degree relatives who are alcoholic.

The acetylator phenotype was determined after a single dose of sulfamethazine by measurement of blood and urine concentrations of sulfamethazine and its acetylated metabolite. These were quantified by high pressure liquid chromatography.

The acetylator phenotype has been determined in a total of 37 alcoholics and 26 unrelated normal volunteers. Both groups contained 54% fast acetylators (20/37 and 14/26). Power analysis indicates that 2430 subjects would be required for these proportions to represent a statistically significant difference.

This study has been terminated.

PROJECT DESCRIPTION

Investigators:

E. Lane Senior Staff Fellow LCS, NIAAA

Objectives:

Any coincidence of the slow acetylator phenotype and alcoholism will be investigated in this study.

Methods Employed:

Acetylator phenotype was measured in unrelated, age and sex matched, normal volunteers and recovered alcoholics. The recovered alcoholics had a history of alcoholism and the normal volunteers were nonalcoholics and had no first degree relatives who are alcoholic.

The acetylator phenotype was determined after a single dose of sulfamethazine by measurement of blood and urine concentrations of sulfamethazine and its acetylated metabolite. These were quantified by high pressure liquid chromatography.

Major Findings:

There is no difference in the distribution of rapid and slow acetylator phenotypes amongst abstinent alcoholics compared to nonalcoholic volunteers.

Significance to Biomedical Research and the Program of the Insititute:

There are two possible points of association between acetylation phenotype and alcoholism. (1) Recent alcohol consumption can alter the "apparent" acetylator phenotype of an individual by increasing his/her acetylation clearance. (2) Acetylation phenotype is, and alcoholism may be, genetically determined and the expression of alcoholism may depend upon genetic factors related to acetylation.

If there should be any coincidence of acetylator phenotype and alcoholism, then further avenues of research in alcoholism will be undertaken.

Proposed Course:

The current study has been terminated.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00255-04 LCS

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.)

Application of Pharmacokinetics to Neurotransmitter Disposition

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

PI:	E. A. Lane	Senior Staff Fellow	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Biochemistry and Pharmacology

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	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 model for the disposition of centrally and peripherally produced norepinephrine has been used to design experiments to separately quantify the central and peripheral production rates of norepinephrine in vivo. Analytical methods required for the execution of these studies have been established in this laboratory. These include the measurement of norepinephrine, normetanephrine, 3-methoxy-4-hydroxy phenylglycol and vanilmandelic acid by gas chromatography-mass spectrometry. Preliminary experiments in animals have demonstrated that some of the analytical methods must be further refined in order to serve our experimental design.

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 AA 00235-06 LCS

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.)
Metabolic and Structural Studies of Polyunsaturated Lipids in Cell Membranes

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

P.I.:	N. Salem, Jr.	Section Chief	LCS, NIAAA
Others:	J. Yergey	Senior Staff Fellow	LCS, NIAAA
	J. Karanian	Senior Staff Fellow	LCS, NIAAA
	H. Kim	Staff Fellow	LCS, NIAAA
	T. Shingu	Visiting Fellow	LCS, NIAAA
	F. Hullin	Visiting Fellow	LCS, NIAAA
	A. Yoffe	Chemist	LCS, NIAAA

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Analytical Chemistry

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.8	4.8	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 principal objective of this study is to elucidate the structural and metabolic functions of polyunsaturated fatty acids or phospholipids with particular reference to their modulation by ethanol. Several approaches to this problem were taken including studies of cellular lipid composition, membrane asymmetry, fatty acid oxygenation and dietary supplementation. In particular, these studies focused on the major polyunsaturate of brain, docosahexaenoate (C22:6w3) and, to a lesser extent, on arachidonate (C20:4w6).

The characterization of the enzymatic oxygenation of docosahexaenoate (22:6w3) was further characterized as a lipoxygenase system. Human platelets produce 11(S), 14(S)- and 17(S)-hydroxy-22:6w3 when the free fatty acid is added to a cell suspension. This reaction is blocked by lipoxygenase inhibitors but stimulated by alcohol. Similar products are also generated in the nervous system in vivo and this metabolite formation is blocked by lipoxygenase inhibitors but stimulated by cerebral ischemia. The 14(S)-hydroxy-22:6w3 is a fairly potent contractile agent on smooth muscle preparations and can antagonize the contractile action of a thromboxane agonist.

Alcohol inhalation decreases the level of platelet 20:4w6 unless a source of dietary 18:3w6 is present. A sensitive method for aminophospholipid molecular species analysis has been developed to follow these leads in terms of both compositional and topographic analyses in alcoholics.

PROJECT DESCRIPTION:

Investigators:

N. Salem, Jr.	Section Chief	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA
J. Yergey	Senior Staff Fellow	LCS, NIAAA
H. Kim	Staff Fellow	LCS, NIAAA
T. Shingu	Visiting Fellow	LCS, NIAAA
F. Hullin	Visiting Fellow	LCS, NIAAA
A. Yoffe	Chemist	LCS, NIAAA
M.B. Engler	Graduate Student	Georgetown Univ.
M.M. Engler	Graduate Student	Georgetown Univ.

Objectives:

(1) In general, to enumerate the various functions of polyunsaturated lipids particularly with reference to excitable membranes and to the chemical transfer of information between cells, with the objective of applying the information obtained to a better understanding of the mechanism of action of ethanol. (2) To elucidate the molecular composition, associations and topographic arrangement of polyunsaturated phospholipids in cellular membranes. (3) To discover and characterize a novel metabolic system of lipoxygenase and/or cyclooxygenase enzymes in mammalian tissues which operates on omega-3 fatty acids. (4) To test the efficacy of dietary supplementation with various classes of polyunsaturated fatty acids in preventing lipid deficiencies in the brain and other organs.

Methods Employed:

Synthesis of Hydroxylated-Docosahexaenoates

Washed platelets were prepared in a TRIS-EDTA buffer and incubated for 20 min at 37°C with 1-100uM of 22:6w3 containing 0.25 uC of [^{14}C]-22:6w3. Rat brains were homogenized in 50mM TRIS, pH 7.4, at 37°C in the presence of 25uM [^{14}C]-22:6w3 for 15 min. In some cases rats had been previously exposed to alcohol inhalation for 7 days in specially designed chambers and using an alcohol vapor concentration of 25 mg/L so as to achieve a mean blood alcohol concentration of 158 mg%. Docosahexaenoic acid (22:6w3) was autooxidized by exposing a thin film of the lipid spread on a round bottomed flask to oxygen for 2 days. Both 5- and 15- lipoxygenase products of 22:6w3 were produced by incubating the fatty acid with potatoe or soybean lipoxygenase, respectively. The effects of various agents on such activity were studied by their addition *in vitro* and these include alcohol, enzyme inhibitors and cofactors or stimulating agents. Reactions were terminated by acidification to pH 3 with formic acid and the metabolites were extracted with equal volumes of dichloromethane.

For study of the *in vivo* metabolism of 22:6w3, rats were anesthetized with chloral hydrate and 5 ul of a saline solution containing 80 umoles of [^{14}C]-22:6w3 was injected into the lateral ventrical (coordinates used were: 1.3mm caudal, 1.9mm lateral and a 3.5mm depth from bregma) using a stereotactic device. Two hours after injection, rat brains were removed and homogenized in methanol and eluted from a C-18 cartridge with ethyl acetate.

Analysis of Hydroxy-docosahexaenoates
Various positional isomers of hydroxy-docosahexaenoates (HDHE) were separated using an HP-1090 HPLC equipped with a diode array UV detector. A 4.6 x 25 cm Axxichrom ODS column (5 um particle size) was used with a flow rate of 1 ml/min and a solvent system composed of 100 mM ammonium acetate and acetonitrile. Autooxidized products of 22:6w3 were separated on a similar column but at 0.8 ml/min, with methanol as the organic modifier and with thermospray ionization (180-186°C at the vaporizor tip) and detection using an Extrel ELQ-400-2 quadrupole mass spectrometer.

GC/MS was used for structural analysis of metabolites in the electron impact mode on an HP-5970B instrument. Hydroperoxy-compounds were reduced to hydroxy-derivatives using sodium borohydride and then methylated with ethereal diazomethane. Trimethylsilyl ethers were prepared using BSTFA in acetonitrite. These derivatives were injected onto an HP-1 methyl silicon column with oven, injector and detector temperatures of 220, 230 and 250°C, respectively, and with helium as carrier gas at a linear velocity of 27 cm/sec. Stereochemical analyses were performed of the methyl esters using a dinitrobenzoxylphenylglycine chiral phase column (5 um, 4.6x25 cm) with a mobile phase of hexane:2-propanol: acetonitrile (497.5:2.5:0.5) and a flow rate of 0.8 ml/min.

Aminophospholipid Molecular Species Analysis
Red cells were incubated for 30 min at 20°C with 2 mM trinitrobenzenesulfonic acid (TNBS) in a buffer containing 120 uM $NaHCO_3$ and 40 mM NaCl, pH 8.5. The lipids were extracted using the technique of Bligh and Dyer. Alternatively, cells were extracted and reacted with TNBS in organic solution in order to label all aminophospholipid species. Trinitrophenyl-phosphatidylethanolamines (TNP-PE) were separated from unlabelled PE and other lipids by TLC using Whatman PLK-5 plates and chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5). TNP-PE species were then eluted in methanol, concentrated, filtered and separated by HPLC. An Axxichrom ODS column (5um, 4.6x25 cm) with a mobile phase of 10 mM ammonium acetate, pH 5.0, and methanol at a flow rate of 1 ml/min and a column temperature of 40°C. Gradients were employed which started at 84% and ended at 93% of organic phase.

Smooth Muscle Bioassays
Guinea pig, rat and rabbits were used as a source of lung parenchymal strips and the latter two species as a source of aortic rings. Animals were killed by cervical dislocation, the preparations trimmed at 4°C and mounted in a 40 ml bath in a Krebs-bicarbonate buffer. Changes in tension were monitored by use of force displacement transducers. The perfusates of these smooth muscle preparations were in some cases collected for eicosanoid assay.

Major Findings

The presence of oxygenated metabolites of 22:6w3 was observed in mammalian tissue *in vivo* for the first time in these experiments. Radiolabelled 22:6w3 was injected into rat brain and HPLC analyses indicated that the labelled components comigrated with previously identified compounds i.e., the 17- , 14- , 11- and 7-hydroxy derivatives of 22:6w3. These compounds were prepared by HPLC after use of enzymatic and autooxidation reactions, GC/MS/EI analyses revealed

that the positions of the hydroxyl moeity could be discerned from the EI spectra. These characteristic ions were then used to monitor the production of these compounds in small amounts by rat brain homogenate *in vitro*. In this way, the presence of 14- and 11-hydroxy-22:6w3 derivatives was verified in the brain homogenate incubation mixtures. The identity of other positional isomers of HDHE's were confirmed on a chiral phase HPLC column as well. The production of these compounds *in vitro* was not affected by cyclooxygenase, monooxygenase or thromboxane synthase inhibitors such as indomethacin, SKF525A or CV-4151, respectively, but were inhibited by lipoxygenase inhibitors such as eicosatetraynoic acid, baicalein, esculetin, nordihydroguariaretic acid (NDGA) and AA-861. However, preliminary studies of the sterospecifcity of these reactions indicated approximately equal amounts of the R and S isomers. Although the exact mechanism through which these compounds are generated in brain is still the subject of ongoing studies, it is nevertheless apparent from our studies that these compounds are formed in mammalian brain, *in vivo*. A three hour ligation of both common carotid arteries in SHR rats led to an increase in production of the various HDHE's produced *in vivo* by 31 to 141%. The biological ramifications of the production of the oxygenated derivatives is therefore of interest. The 12-lipoxygenase of human platelets was also intensively investigated for its activity towards 22:6w3 for possible application to clinical assays. Careful analyses of the products formed by platelets by reversed phase and chiral phase HPLC, by LC/MS and GC/MS indicated that 14(S)hydroxy-22:6w3 was the principal product but that 11(S)- and 17(S)-hydroxy 22:6w3 were also produced. Lipoxygenase inhibitors such as ETYA (1uM) and NDGA (40uM) effectively blocked the production of HDHE's.

The platelet is believed to possess a 15-lipoxygenase, however, this activity could also be due to a small number of contaminating cell types (eg. granulocytes). The stereoselective 12-lipoxygenase activity of platelets was also observed in other species and the activity was observed in the following order: man > rat > guinea pig > dog > monkey > rabbit. Rats could therefore be used to investigate the effects of chronic alcohol intake on this aspect of polyunsaturate metabolism. After seven days of alcohol inhalation, rat platelets produced 178% more 14(S)-hydroxy-22:6w3 *in vitro*. This was consistent with our findings that ethanol inhalation lowered polyunsaturate and, in particular, 20:4w6 levels in rat platelets when animals were fed an 18:1w9 or 18:3w3 enriched diet. However, increasing the level of 18:3w6 in the diet prevented the loss of platelet 20:4w6 from alcohol exposed animals. These findings may have important implications for alcoholic patients and suggest that increased conversion of cellular polyunsaturates into oxygenated metabolites may be associated with common pathological changes observed in these patients.

The metabolites formed are biologically active as they are capable of inducing airway smooth muscle contraction; in the lung parenchymal strips, an equimolar concentration of a thromboxane-agonist, U46619, was 10-20-fold more efficacious than HDHE. HDHE may act in part through stimulation of leukotriene production as increased peptidyl-leukotriene levels (152%) were associated with the HDHE-induced contraction in this preparation and NDGA was capable of a partial blockade of this response. In addition, HDHE antagonizes the contractile effects of the thromboxane-agonist but not that of norepinephrine in the vascular (rabbit and rat aorta), airway (guinea pig lung parenchyma) and visceral

(rat stomach strip) smooth muscle preparations studied. An anti-thromboxane activity of some specificity may therefore be one property of HDHE. Furthermore, our studies indicated that the 15-lipoxygenase derivative of DHA, i.e., 17-HDHE, is also active on these preparations, whereas some eicosanoids (12-HETE, 12-HPETE and 12-HEPE) are not.

Another area of investigation was the possible direct action of 22:6w3 fatty acid on smooth muscle contraction. A concentration-dependent relaxation of rat aortic strips was observed in the 1-64 uM range when the sodium salt of 22:6 was added to the baths. Indomethacin and NDGA preincubation had no effect on this phenomenon ruling out eicosanoids as mediators of the 22:6w3 effect on muscle tone. These observations were generalized to other fatty acids as well in this reporting period. It was observed that 18:2w6, 20:4w6 and 20:5w3 but not w-9 fatty acids induced a decrease in rat aorta tone and all of these effects, with the exception of 20:4w6, were independent of eicosanoid production as assessed by the use of the inhibitors indomethacin and NDGA. The relaxant effects of 22:6w3 and 20:5w3 were not affected by the removal of the endothelium.

Progress has been made in the development of a sensitive and detailed method for the study of aminophospholipid species composition and localization in the plasma membranes of blood cells. The development of gradient conditions has improved the separation of TNP-PE species so that more accurate estimates of the amount of each of them can be obtained. Quantitative aspects of this assay have been studied and it has been shown that all species within a single phopholipid class have the same response factors. Furthermore, a linear response of the UV signal at 338 nM is obtained for 0.1 to 10 nmoles of TNP-PE's. These factors, in combination with the use of internal standards, will allow the expression of data as moles per ml of packed red cells or as a mole percentage report. The detection limit was 10 pmoles. Recovery experiments indicated that no components are selectively lost and also a good overall recovery for PE's. Analyses of human red cells indicate that monoenoic species of PE are more often found on the cell surface and that polyunsaturated ones are preferentially localized on the cytoplasmic leaflet of the plasma membrane. This phospholipid molecular species asymmetry can now be studied in alcoholic patients.

Significance to Biomedical Research and the Program of the Institute:

The discovery of a leukotriene-like lipoxygenase product formed by rat brain in vivo is a significant development for the elucidation of the functions of polyunsaturates in brain. This is the first description of 22:6w3 metabolism in vivo in any mammalian tissue. The demonstration of the stimulation of this pathway in brain by cerebral ischemia or by alcohol in platelets indicates that it is subject to physiological modulation and may be a biological response to toxic stimuli. The loss of membrane polyunsaturates and the increased production of their oxygenated metabolites may be an important mechanism mediating the pathological effects of alcohol abuse. These were also the first studies demonstrating that the metabolites of 22:6w3 have biological activity on smooth muscle preparations.

Proposed Course:

We will continue our characterization of membrane microenvironments with respect to polyunsaturate localization and the effects of alcohol on these environments. Human red blood cell samples will be analyzed to establish whether alcoholics are abnormal in this respect. Nutritional studies will be extended to determine the effects of omega-3 fatty acids in various tissues with respect to both compositon and localization and to determine whether the effects of alcohol can be reversed by such treatment. Thermospray LC/MS studies will undergo further basic development in order to develop facile methods for quantification of molecular species composition in complex biological samples. Docosahexaenoate metabolism studies in rat brain *in vivo* will be extended to determine whether alcohol inhalation alters the rate or route of metabolism.

Pilot experiments will also be undertaken in order to see whether the platelets of alcoholics also have increased 12-lipoxygenase activity and decreased levels of polyunsaturates.

Publications:

Palmblad J, Wannemacher RW, Salem N Jr, Kohns DB, Wright DG. Essential fatty acid deficiency and neutrophil function: studies of lipid-free total parental nutrition in monkeys, J Lab Clin Med 1988;111:634-44.

Salem N Jr, Kim H-Y, Yergey JA. Thermospray-liquid chromatography/mass spectrometry of eicosanoids and phospholipids. In: Lagarde M. ed. Collogue Inserm. Biology of Eicosanoids 1987;752:151-62.

Shingu T, Salem N Jr. Role of oxygen radicals in peroxidation of docosahexaenoic acid by rat brain homogenate *in vitro*. Walden TL Jr, Hughes HN, eds. Prostaglandins and Lipid Metabolism in Radiation Injury, Plenum Press, NY 1987;103-8.

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Karanian JW, Salem N Jr. The effect of alcohol inhalation on the cardiovascular state of the rat, Adv Alc Subst Abuse, in press.

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Kim H-Y, Karanian JW, Salem N Jr. Structural analysis of oxygenated metabolites of polyunsaturated fatty acids using thermospray LC/MS and GS/MS, Ann NY Acad Sci, in press.

Karanian JW, Kim H-Y, Shingu T, Yergey JA, Salem N Jr. Smooth muscle pharmacology of hydroxylated docosanoids, NY Acad Sci, in press.

Shingu T, Karanian JW, Kim H-Y, Salem N Jr. Lipid peroxidation of docosahexaenoic acid in rat brain in vitro and in vivo, Ann NY Acad Sci, in press.

Salem N Jr, Shingu T, Kim H-Y, Hullin F, Bougnoux P, Karanian JW. Specialization in membrane structure and metabolism with respect to polyunsaturated lipids, In: Karnovsky M, Leaf A., eds. Aberrations in Membrane Structure and Function, Liss Alan R, New York, in press.

Salem N Jr. Omega-3 fatty acids: molecular and biochemical aspects, In: Spiller G. ed. New Protective Roles of Selected Nutrients in Human Nutrition, Liss Alan R, New York, in press.

Salem N Jr, Hullin F, Yoffe AM, Karanian JW, Kim H-Y. Fatty acid and phospholipid species composition of rat tissues after a fish oil diet. In: Wong P, Samuelson B, Sun F., eds. Advances Prostaglandin Leukotriene Res, Raven Press, New York, in press.

Karanian JW, Kim H-Y, Shingu T, Yergey JA, Salem N Jr. Smooth muscle effects of hydroxylated docosahexaenoates produced from human platelets, Biochem Biomed Acta, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00251-04 LCS

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 Role of Prostaglandins in Mediating the Effects of Alcohol on Smooth Muscle

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

PI:	N. Salem, Jr.	Section Chief	LCS, NIAAA
Others:	J. Karanian	Senior Staff Fellow	LCS, NIAAA
	T. Shingu	Visiting Fellow	LCS, NIAAA
	A. Yoffe	Chemist	LCS, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Analytical Chemistry

INSTITUTE AND LOCATION

NIAAA, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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.)

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00262-04 LCS

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.)*
Characterization of oxygenated fatty acid metabolites by capillary GC/MS.

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

PI:	J. Yergey	Senior Staff Fellow	LCS, NIAAA
Others:	N. Salem, Jr.	Section Chief	LCS, NIAAA
	H. Kim	Staff Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA

COOPERATING UNITS *(if any)* Clinical Neuroscience Branch, NIMH (D. Pickard); Lab. Clinical Science, NIMH (M. Rudorfer); Neuropsychiatry Branch, St. Eliz. Hosp. (C. Kaufmann); Dept. of Neurology Services, Veterans Hosp., Wash., D.C. (J. Hawley), Laboratory of Neurophysiology, NIMH (M. Heyes)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Analytical Chemistry

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

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

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

The objective of this project has been to develop and apply a sensitive and specific gas chromatographic / mass spectrometric assay for eicosanoids in in human cerbrospinal fluid (CSF) to alcohol patients and appropriate controls. This study was initiated because numerous literature reports suggested that a relationship exists between the central nervous system effects of ethanol and the central production of prostaglandins. In both human and animal studies, administration of prostaglandin synthesis inhibitors prior to administration of ethanol has been shown to attenuate central nervous system effects of ethanol.

Prostaglandin concentrations in lumbar CSF of healthy humans and abstinent the alcoholics, and in cisternal CSF of Rhesus monkeys are below 5 pg/mL CSF for the five prostaglandins measured (PGE_2, PGE_1, PGF_{2a}, PGF_{1a}, 6-keto-PGF_{1a}). literature reports of much higher levels of prostaglandins in healthy humans, e.g., tens to hundreds of pg/mL, are due to methodological shortcomings.

PROJECT DESCRIPTION:

Investigators:

J. Yergey	Senior Staff Fellow	LCS, NIAAA
N. Salem, Jr.	Section Chief	LCS, NIAAA
H.Y. Kim	Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Pickard	Section Chief	NSB, NIMH
M. Rudorfer	Staff Psychiatrist	LCS, NIMH
C. Kaufman	Medical Staff Fellow	St. Eliz. Hosp.
J. Hawley	Staff Neurologist	Veterans Hosp., Wash., D.C.
M. Heyes	Visiting Associate	LN, NIMH

Objectives:

The specific aims of this study were: 1) To elucidate aspects of the molecular mechanisms underlying neuropathological states, with particular emphasis on alcoholism and the role of oxygenated fatty acid metabolites; 2) To develop analytical methodologies which can be utilized for quantification of trace oxygenated fatty acid metabolites in various biological fluids; and 3) to develop a sensitive and specific assay for prostaglandins in human cerebrospinal fluid with the goal of correlating their levels with clinical characteristics of patients.

Methods employed:

Sample collection:
Human lumbar cerebrospinal fluid samples, the 22-23 mL of a 30 mL spinal tap, were drawn on ice into polypropylene tubes. Exactly 2 mL was transferred to a second tube containing internal standards (250 pg each of tetradeuterated PGE_2, PGF_{2a},and 6-keto-PGF_{1a}), and tubes were frozen at -70°C. Rhesus monkey CSF samples were acquired in order to investigate another closely related source of CSF, which had the possibility for larger sample sizes and timed profiles. Rhesus monkey lumbar CSF samples were drawn from an indwelling catheter. Sampling began after ketamine anesthesia wore off and continued for 3-4 days. Rhesus monkey cisternal CSF was drawn following administration of ketamine anesthesia. Internal standards were added to monkey samples just prior to derivatization and extraction.

Extraction, Derivatization and GC/MS Determination:
Thawed samples were treated overnight with twice the sample volume of 1% methoxylamine-hydrochloride in phosphate buffer (pH 5-7). Solid phase extraction using 1 mL Bond-Elut cartridges proceeded as follows: aqueous samples were applied to conditioned cartridges, rinsed with 2 mL of 20% methanol, dried for 5-10 min under vacuum, rinsed with 2 mL of dichloromethane, and the prostaglandins eluted in 2 mL of methanol directly into silanized glass vials. Extracted samples were further derivatized to the

pentaflurobenzyl ester, trimethysilyl ether, and assayed by capillary gas chromatography / electron-capture negative ionization mass spectrometry using on-column injection. The mass spectrometer was operated in selectedion-monitoring mode, and quantification was made by comparison of peak areas for the prostaglandins to their respective tetradeuterated internal standards.

Major Findings:

Assay Development:
Developments in the assay procedure have significantly reduced contamination from the derivatization reagents, and have allowed much shorter GC/MS cycle times. Aqueous methoximation reaction allows the byproducts of this reaction to be separated in the extraction step, and eliminates the use of pyridine. Dichloromethane has replaced the more dangerous benzene for removal of non-polar compounds in the extraction step. Using dodecane as the final sample solvent allowed the GC/MS determination to be completed in less than seven minutes rather than the previous twenty minute analysis time.

Prostaglandin Concentrations in Human and Rhesus Monkey CSF:
The concentrations of prostaglandin E_2, E_1, F_{1a}, F_{2a}, and 6-keto-F_{1a} in lumbar CSF from healthy human volunteers and alcoholics following 3 weeks abstinence (n=37), and in Rhesus monkey cisternal CSF were below the limit of quantification of 5 pg/mL CSF. These results indicate that either prostaglandin production or transport of the prostaglandins in the central nervous system limits their concentration in the bulk CSF. In contrast, lumbar CSF from Rhesus monkeys was found to contain greater than 200 pg/mL of PGE_2, PGF_{2a}, and 6-keto-PGF_{1a}. This latter result was probably due to local production near the site of the indwelling catheter.

Significance to Biomedical Research and the Program of the Institute:

These studies have shown that very low levels of prostaglandins are present in human cerebrospinal fluid. Because this is in conflict with many literature reports of higher levels in healthy humans, it indicates that future studies of prostaglandins in human CSF should include samples from healthy volunteers in which appropriately low levels are verified.

Proposed Course:
Study of human CSF will center on measurements of prostaglandin concentrations in CSF of drinking alcoholics and alcoholics during acute withdrawal, where levels are more likely to be elevated. Expansion of efforts to include measurement of other arachidonic acid metabolites and other bodily fluids are also planned.

Publications:

Yergey JA, Salem Jr, N, Karanian JW, Linnoila M. GC/MS Assay of Prostaglandins in Cerebrospinal Fluid from Humans and Monkeys, Adv Alcohol Sust Abuse, in press.

Yergey JA, Salem Jr, N, Karanian JW, Linnoila M, Heyes MP. Prostaglandins in Human Cerebrospinal Fluid?, Ann NY Acad Sci 1988, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00252-05 LCS

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 Effect of Ethanol on POMC Peptide Synthesis and Release In Vivo and In Vitro.

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

PI:	R. Eskay	Physiologist	LCS, NIAAA
Others:	A. Thiagarajan	Visiting Fellow	LCS, NIAAA

COOPERATING UNITS (if any)
Laboratory of Cell Biology, NIMH (M. Grino)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION

INSTITUTE AND LOCATION
NIAAA, 9000 Rockville Pike, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

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

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

The administration of ethanol is known to alter certain regulatory aspects of the hypothalamic-pituitary-adrenal axis (HPAA) and since the integrity of this system depends on the synthesis and secretion of specific regulatory neuropeptides at the hypothalamic (e.g., corticotropin-releasing hormone (CRF); vasopressin (AVP)) and pituitary gland (e.g., beta endorphin (BE); ACTH) level, we have been evaluating the impact of ethanol on the HPAA as a model system _in vivo_ and _in vitro_. In order to discern the direct versus indirect effects of ethanol at the cellular level, we have been working with AtT-20 cells, corticotropes, and melanotropes in culture and ethanol-exposed intact male rats. It is envisioned that the exploration of each of the component parts of the HPAA (i.e., CNS, pituitary and adrenal gland) independent of one another and the subsequent intergration of results from the intact animal will enable profiling the acute, chronic, direct and indirect effects of ethanol at the cellular and subcellular level, as well as the primary versus secondary sites of ethanol-induced perturbations of the HPAA. Since neuroendocrine cells are continuously synthesizing and secreting products, our studies in their broadest sense are designed to understand the effects of ethanol on stimulus-secretion-synthesis coupling in secretory cells. To date our investigations have yielded a variety of information.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION:

Investigators:

R. Eskay	Physiologist	LCS, NIAAA
A. Thiagargan	Visiting Fellow	LCS, NIAAA
M. Grino	Visiting Fellow	LCB, NIMH

Objectives:

The ongoing aims of this group of experiments are: 1) to study the biosynthesis and regulation of release of neuropeptides from the pituitary gland, CNS and tumor cell lines, which includes an understanding of the sequence of events from membrane-receptor activation to intracellular-messenger systems to physiological responses, 2) to determine the concentration-dependent and time-dependent effects of ethanol on the various cellular events as outlined in 1, both *in vivo* and *in vitro* and 3) to elucidate the consequences of certain indirect effects of ethanol on the HPAA, which are mediated via chronic corticosterone elevation.

Methods Employed:

AtT-20 cells, an anterior pituitary derived tumor cell line, and dispersed anterior pituitary (AP) and intermediate lobe (IL) cells, which synthesize and secrete POMC-neuropeptides, were cultured. Prior to the one hour incubation of cells with various secretogogues with or without ethanol, the culture medium was removed and the cells were preincubated for up to 48 hours in the presence of ethanol. Following the 1-4 hour experimental incubation,the medium was removed and centrifuged to remove detached cells. The supernatant fluid was obtained for the determination of beta-endorphin and cyclic AMP by radioimmunoassay. In certain experiments the binding of [I^{125}]-corticotropin-releasing hormone to and POMC messenger RNA (mRNA) in cultured cells was evaluated. In other experiments, animals received either a single dose or multiple doses of ethanol via a indwelling intragastric cannula for up to 7 days.

Major Findings:

Effect of acute or chronic ethanol exposure on the HPAA in vivo

In a previous study, animals exposed continuously to ethanol vapors for up to 14 days in an inhalation chamber with continuous blood ethanol levels between 100-250 mg% were found to have 25-30% lower CRF binding capacity and basal AC activity in membranes from endocrine tissue (e.g. AP, IL and adrenal membranes) and non-endocrine peripheral tissue (i.e., liver and kidney). In addition chronic ethanol treatment rendered CRF-responsive tissue, as determined by monitoring membrane-associated adenylate cyclase activity, refractory to subsequent CRF stimulation. Chronic ethanol exposure resulted also in a time-related decrease in POMC mRNA levels in both the AL and IL of the pituitary gland. IL lobe POMC mRNA levels, which reflect the biosynthetic activity of cells expressing this message, are more sensitive to ethanol exposure than AP-POMC mRNA levels. This result would argue that ethanol's effects, at least on IL cells, are direct and not influenced by elevated glucocorticoid levels, since glucocorticoids do not significantly alter IL cell functions.

Chronic exposure of adult male rats to continuous blood ethanol levels of 150-350mg% via multiple dose ethanol administration for up to 7 days has enhanced our understanding of ethanol's effects on the HPAA. Continuous ethanol exposure resulted in asynchrony between plasma ACTH and corticosterone (CS) levels. ACTH levels following 12 hr, 1 day or 3 days of continuous ethanol exposure did not differ from non ethanol treated animals; however, plasma CS levels were elevated at all times. Further treatment for up to 7 days resulted in a return of CS to control levels. The observed reduction in median eminence CRF at 7 days ethanol exposure and no change in AVP median eminence levels, suggests that CRH metabolism but not AVP activity is altered.

In order to explore certain of the apparent contradictions between the acute and chronic effects of ethanol on the HPAA, adult male rats received a single infusion of ethanol via a chronic indwelling intragastric catheter followed by the determination of plasma ACTH, CS and catecholamines (epinephrine (EPI), norepinephrine (NOREPI)). Blood ethanol concentrations determined at 15,30,60, 90 and 150 min. post-ethanol infusion were similiar at all sample times and ranged from 240-280 mg%. Following ethanol infusion, plasma EPI levels increased 6-10 fold at 15 min and returned to basal levels by 60 min. NOREPI levels increased approximately 2 fold by 15 min. post-ethanol infusion and remained elevated for up to 2 hrs. The maximal observed increases in ACTH and CS levels were 10 and 4-fold respectively. Since stress hormone secretion is under multifactorial control, the same would seem logical for ethanol activation of the HPAA. However, administration of vasopressin antiserum to neutralize median eminence AVP or removal of plasma EPI through adrenal demedullation, does not significantly blunt plasma ACTH peaks following a single-dose ethanol challange. These results suggest that neither hypophsial AVP or medullary derived EPI are significant co-regulators of ethanol activation of the HPAA.

Effect of acute or chronic ethanol treatment on cultured AtT-20 and pituitary cells in vitro.

Incubation of AtT-20 or AP cells in the presence of ethanol (0.4%) for less than 48 hrs has only a marginal effect on basal or secretogogue-induced BE release and cAMP levels; however, incubation of both cell types in the presence 0.4% ethanol for 48 hrs. or longer results in a reduction of basal and secretogogue (CRF, isoproterenol) induced cAMP levels and BE secretion. In contrast to the observed ethanol-induced inhibition of the AC/cAMP-mediated secretogogue response, the ability of the calcium ionophores ionomycin or A23187 or 50mM K^+ to enhance BE secretion from AP cells was not altered by preincubation in the presence of ethanol. It would appear that chronic ethanol treatment alters AC/cAMP mediated BE secretion, and that enhanced intracellular Ca^{++} levels can override ethanol's inhibition of BE secretion.

In addition to the observed effects of ethanol on BE secretion, ethanol produced a significant decrease in POMC mRNA levels in, AP and IL cells only after 48 hrs of ethanol treatment at 0.4%. Although ethanol directly inhibits POMC mRNA synthesis in each cell type studied, the specificity of this inhibition is is currently under investigation. Finally in five independent experiments neither the dissociation constant (Kd, 2.6 ± 0.6nM) nor the receptor concentration (B_{max}, 70-80 fmoles/mg protein) of CRF binding sites varied with ethanol treatment. This suggests that ethanol's ability to inhibit CRF-induced hormone secretion *in vitro* lies beyond the level of membrane, receptor activation.

Significance to Biomedical Research and the Program of the Institute:

The continued exploration of the effects of ethanol on fundamental cellular events, utilizing well-characterized in vivo and in vitro model systems should provide an understanding of the multiple, ethanol-induced perturbations of normal cellular functions. This, in turn, will hasten the development of effective therapeutic agents to treat patients with alcohol-related dysfunctions.

Proposed Course:

The in vivo studies are continuing with an emphasis on delineating the effects of continuously elevated glucocorticords on CNS glucocorticord receptor types and number in various brain regions. The observation that selected enrichment of culture media reduces ethanol's overall toxic effects on secretory cells in vitro is also being explored.

Publications:

Thiagarajan A, Mefford I, Eskay R. Acute effect of intragastric ethanol adminisatration on plasma levels of stress hormones, Adv Alc Subst Abuse 1988;7:227-31.

Palkovits M, Eskay R, Antoni F. Atrial natruiretic peptide in the median eminence is of paraventricular nucleus origin, Neuroendocrinology 1987; 46:542-4.

Dave JR, Eskay RL. Ethanol decreases cortiotropin-releasing factor binding adenylate cyclase activity, pro-opiomelanocortin biosynthesis, and beta-endorphin release in cultured pituitary cells, Ann NY Acad Sci 1987;492:327-30.

Waschek J, Eskay R, Eiden L. Barium distinguishes separate calcium targets for synthesis and secretion of peptides in neuroendocrine cells, Biochem Biophys Res Commun 1987; 146:495-501.

Kiss A, Palkovits M, Antoni F, Eskay R, Skirboll L. Neurotensin in the rat median eminence: the possible sources of neurotensin-like fibers and varicosities in the external layer, Brain Res 1987;416:129-35.

Thiagarajan A, Mefford I, Eskay R. Single dose ethanol administration activates the HPAA: Exploration of the mechanism of action, Neuroendocrinology, in press.

Annual Report of the
Laboratory of Metabolism and Molecular Biology
Division of Intramural Clinical and Biological Research
National Institute of Alcohol Abuse and Alcoholism
October 1, 1987 to September 30, 1988
Richard L. Veech, M.D., D.Phil., Chief

Introduction

The laboratory is divided into three complementary sections concentrating respectively on the techniques of molecular biology, NMR spectroscopy and studies of metabolic control. The objectives and research emphases of each of the three sections are summarized below. The Section of Physical Chemistry, the newest of these sections, was organized during this past year. Most of the energies of this section during the past year were devoted to installing necessary equipment and recruiting additional staff members needed to undertake the proposed work.

I. Section of Molecular Genetics

This section is continuing to do research in three major areas of interest which are summarized as follows:

a. The mechanism of enzymatic changes induced by chronic ethanol ingestion:

For almost twenty years, a subject of continuing interest in alcohol research has been the observation that chronic ethanol ingestion leads to the hypertrophy of hepatic microsomes. For many years it was widely held that this microsomal induction resulted in:

1. an increase in the proportion of ethanol metabolized by the "microsomal ethanol oxidizing system;" and

2. an increase in the steady state level of the metabolic product of this reaction, acetaldehyde, which explained much of the resultant toxicity associated with chronic alcoholism; and

3. the hypertrophy of this microsomal system leading to abnormal drug metabolism in alcoholics and an increased risk of malignancy due to the conversion of many xenobiotics into active carcinogens. Work in this laboratory over the last several years has established that while microsomal ethanol oxidation does not account for significant amounts of ethanol metabolism, the elevation of the previously unknown human metabolite, 1,2-propanediol in the blood of a majority of alcoholic patients results from the conversion of

ketone bodies to 1,2-propanediol. The enzyme responsible for the rate limiting step in this pathway is the ethanol inducible form of P_{450} named P_{450}IIe. This enzyme was subsequently cloned and the complete intron and exon sequence and relationship to developmental expression described. Investigation in the diabetic rats shows that the increased levels of P_{450}IIe result from post-translational stabilization of the mRNA, whereas feeding of ethanol or acetate results in no increase in the level of mRNA for P_{450}IIe. Other workers using different methods have concluded that the mechanism accounting for increased cytochrome P_{450}IIe alcohol feeding is an increase in the messenger RNA level. Work currently in progress in our laboratory suggests that this is not the case, but rather that the increased levels of P_{450}IIe result from yet a different mechanism, namely a decreased rate of P_{450}IIe degradation.

b. Identification of markers for human alcoholism:

In a blind study of 250 patients at the Royal Free Hospital, London, it was found that 25% of patients with biopsy proven alcoholic cirrhosis had elevated levels of D,D-L,L-2,3-butane-diol in the blood while abstinent from ethanol for up to 16 years. Elevation of D,D-L,L-2,3-butanediol was not found in patients with other forms of liver disease. In the light of previous findings that about 70% of active alcoholics have elevation of blood 2,3-butanediol while drinking distilled spirits, this finding strengthens the conclusion that the presence of elevation of 2,3-butanediol in the blood is specifically associated with severe alcoholism and that the expression of this metabolic marker is potentiated by the ingestion of distilled spirits, or by the development of alcoholic cirrhosis.

The gene product responsible for the presence of 2,3-butanediol in the blood of alcoholics remains to be established. It was hypothesized, based on earlier work in animal systems that abnormalities of one of the subunits of the pyruvate dehydrogenase multienzyme complex might be responsible for this finding. Accordingly, the E_1 alpha, E_1 beta and E_3 subunits of the PDH multienzyme complex have been cloned from human and rat brain and liver over the past year. So far, no clone of the E_2subunit has been obtained. Detailed analysis of the kinetics of the reaction of the E_1 alpha subunit are now underway to determine if abnormalities in this gene product are responsible for the occurrence of elevation of 2,3-butanediol in alcoholics. From this analysis, an assessment of the significance of this finding can be made.

c. The role of cellular growth factors in human alcoholism:

Abnormalities of cell growth, particularly proliferation of fibrous tissue as in alcoholic cirrhosis, are characteristic of many of the pathological states found in chronic alcoholism. Recently it has come to be recognized that proliferation of cell types, or change in the function within the same cell is the result of growth stimulatory and growth inhibitory factors. Many of these factors are small peptides, produced by a variety of cell types, of molecular weights generally in the range of 17 to 36 kD, many of which are gene products of the so-called oncogenes such as myc, sis, fos and others. In the light of these developments, some of the pathological changes long associated with alcoholism can be viewed as representing various stages in cell transformation.

In alcoholism research, great emphasis has historically been placed upon the role of acetaldehyde in the toxicity of ethanol. In contrast, very little attention has been placed upon the effect of the terminal metabolite of ethanol, acetate. In regard to the secretion of growth factors however, acetate has been shown to potentiate the release of such factors from monocytes and macrophages by bacterial lipopolysaccharides and to be capable of inducing the release of such factors in and of itself. Among these peptides are interleukin 1a and b, interleukin 6, platelet derived growth factor, tumor necrosis factor and 11 other factors the majority of which are unidentified as to either structure or activity.

Much of our knowledge about such factors has been derived from studying the action of these peptides upon cells in culture where the reactions seen may or may not represent accurately the changes occurring in vivo. Accordingly the effects of several of these factors have been investigated for the first time in vivo. It was determined that platelet derived growth factor, five minutes after injection into the rat, results in an increase of 2.4 times in the Vmax of glucose 6-phosphate dehydrogenase with no change in Km for NADP or glucose 6-phosphate. Accompanying this change and indicative of it, was an elevation of the intermediates of the hexose monophosphate pathway. As the supply of ribose phosphate is essential for nucleotide synthesis, the relationship of these changes to cell growth and hypertrophy is a subject of further study of some interest. In another study, the acute effects of epidermal growth factor, a peptide secreted by salivary gland and gut, was investigated and found to result within five minutes in a decrease in the Vmax of pyruvate kinase with no change in the Km for phosphoenolpyruvate. Also associated with this change was an unusual increase in the degree of phosphorylation of glucose, by a mechanism which we were unable to relate to kinetic changes in the known enzymes believed to be involved

in this reaction, namely glucokinase or glucose 6-phosphatase.

In a more clinical application of this area of study the effects of some of these factors in alcoholic hepatitis were investigated. This complication of alcoholism is among the most severe pathological consequences of severe alcoholism. The pathophysiological mechanisms involved remain unknown and, as a consequence, there is no satisfactory treatment other than general supportive measures. In a collaborative study with the Johns Hopkins School of Medicine of 25 patients with severe alcoholic hepatitis, about two-thirds of those patients with severe alcoholic hepatitis demonstrated elevated levels of tumor necrosis factor in their blood during hospitalization. The absence of measurable tumor necrosis factor alpha was significantly correlated with survival up to one to two years post discharge, whereas the presence of this peptide in blood was associated with death at a mean period of four months following discharge. None of the other standard liver function tests or clinical signs associated with this disease was significantly correlated with clinical outcome. This finding, therefore, is likely to have important implications, not only for our understanding of the pathophysiological mechanisms underlying this malady but to open up new avenues of therapy in a disease which has a 40% mortality with current treatment modalities.

II. Section of Physical Chemistry

Since the Section on Physical Chemistry was organized during this fiscal year a major portion of the effort of its efforts were devoted to installing its NMR spectrometers at the NIH NMR facility on the main campus and recruiting two senior visiting scientists and one senior staff fellow to initiate the Section's programs. At the same time collaborative studies using in vivo NMR on animals were continued at the University of Pennsylvania. Modification of the NMR apparatus at NIH was undertaken this year to permit studying energy metabolism in the muscles of patients with panic disorder who are being studied in the Laboratory of Clinical Studies. These studies are scheduled to begin during the next fiscal year.

a. Measurement and control of cerebral energy metabolism and cerebral blood flow:

A major effort of this section will be focused on the measurement of cerebral metabolic rate and cerebral blood flow using non-invasive NMR techniques. Preliminary techniques have been developed by Dr. McLaughlin and Dr. Huang. These techniques will be amplified and expanded over the next two years by Drs. Dora, Ligeti and Lyon to investigate the effects of ethanol upon cerebral blood flow and oxygen consumption, and the relationship of these

effects to the levels of cerebral creatine phosphate, ATP and Pi.

Preliminary studies with new blood flow techniques developed in the laboratory show that the results are in good agreement with simultaneous estimations of blood flow using microsphere techniques.

In collaboration with scientists at the University of Pennsylvania, Dr. McLaughlin and Ms. Sinnwell are continuing studies on coupling between cerebral metabolic rates and cerebral blood flow in the cat. In this model, the cerebral blood flow is varied by adjusting intracranial pressure. Preliminary results indicate that cerebral blood flow can be decreased by a factor of three without any change in the cerebral metabolic rate, due to a corresponding increase in the arteriovenous difference for oxygen.

In another aspect of this problem, Dr. Dora is initiating studies of the effects of various pharmacological agents such as propranolol, acetylcholine, indomethacin and ethanol upon vascular endothelium, and the relationship of these effects to the contraction of vascular smooth muscle in isolated arterial preparations. Finally, Mr. Hines is attempting to directly determine plasma levels of free [Mg2+] using electrodes since variations in the concentration of this simple cation have been implicated in hypertension, and depletion of levels of free [Mg2+] are often found in alcoholics undergoing withdrawal.

b. Membrane studies:

There has been a long standing interest in the effects of ethanol upon the physical state of cell membranes and the membrane protein imbedded within the membrane. One aspect of this problem is the effect of charged residues on the electrostatic surface potential of membranes. Those imbedded within the membrane have differing effects than those extending some distance from the surface of the membrane, for example, sialic acid residues. Mr. Hines has developed a method for the incorporation of glycophorin, a sialated glycoprotein component of the red cell membrane, into reconstituted membrane vesicles. The effects of such incorporation on the surface potential of lipid bilayers will be investigated in the coming year.

c. NMR studies of pancreatic metabolism:

Acute pancreatitis is a common and severe complication of chronic alcoholism. Studies were undertaken by Dr. Janes, in collaboration with scientists at Johns Hopkins Medical School, using NMR spectroscopy to investigate the effects of agents which induce pancreatitis in the dog. It was found that agents which induce pancreatitis in dogs were

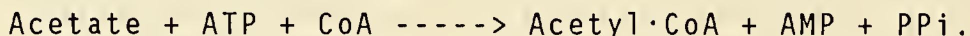
associated with a rapid drop in high energy phosphate intermediates in the pancreas. The results of these studies are in press.

III. Section on Metabolic Control

This section has concentrated its work in four areas: the effects of acetate upon calcium and inorganic pyrophosphate metabolism; the control and determination of intracellular pH and its relationship to cellular phosphorylation potential and work; the relationship of ingested glucose to hepatic glycogen metabolism; and the measurement and control of the intermediates of purine and pyrimidine nucleotide synthesis.

a. The effects of acetate upon calcium and inorganic phosphate metabolism:

The final product of the hepatic metabolism of ethanol is acetate. The metabolism of acetate is accomplished by the reaction:


Acetate + ATP + CoA -----> Acetyl·CoA + AMP + PPi.

Under certain conditions, this can lead to the accumulation of large amounts of inorganic pyrophosphate in tissues, and with this the accumulation of calcium and magnesium, presumably within the mitochondrial matrix as insoluble calcium and magnesium pyrophosphate salts. Associated with these changes are profound changes in the status of cellular energy state, in particular, a drop in the phosphorylation potential, reduction of the free cytosolic NADP+/NADPH ratio and most paradoxically, an oxidation of the mitochondrial NAD+/NADH.

In studies undertaken during the past year in collaboration with scientists at NIDDK and BEIB at NIH, we have shown, using electron microprobe analysis, that the accumulation of calcium which occurs under conditions of acetate metabolism elevates mitochondrial calcium twenty-fold while increasing mitochondrial magnesium about two-fold. From these direct measurements, one may draw the inference that the mitochondrial free [Ca2+] is approximately the same as mitochondrial free [Mg2+] leading to the postulate that the free mitochondrial [Ca2+] is of the order of 10^{-3} M *in vivo*. The magnitude of the gradient of free [Ca2+] from about 10^{-7} M in cytoplasm to 10^{-3} M in mitochondria would be compatible with the observation that administration of acetate causes a paradoxical oxidation of the mitochondrial NAD+/NADH and is further compatible with the conclusion that the primary redox energy of the electron transport chain is in near equilibrium with the gradient of free [Ca2+] across the mitochondrial inner membrane. Application of near-equilibrium thermodynamic considerations of this type raises fundamental issues about the status of total

mitochondrial water. The constancy of the activity of free water throughout all tissue compartments was also addressed.

b. Measurement of intracellular pH, phosphorylation potential and relationship to work:

The use of ^{31}P NMR spectrophotometry has become widespread in the evaluation of tissue phosphorylation potential and pH. Because of the non-invasive nature of this procedure, it is thought to have wide applicability in clinical settings. However, the calibration of such measurements with enzymatic analyses of tissue components has not been rigorously undertaken. Accordingly, a 12 Tesla NMR apparatus was used in collaboration with scientists at the University of Pennsylvania to monitor phosphorylation potential and actual cardiac work during perfusion of hearts followed by enzymatic analysis of tissue components. At the same time evaluation of intracellular pH by NMR and by analysis of tissue levels of bicarbonate and CO_2 was undertaken. An analysis of this data will be completed during this fiscal year and will disclose several significant discrepancies between ^{31}P NMR measurements and those of classical analytical chemistry.

Measurement of tissue bicarbonate using classical Van Slyke gaseometric analysis has shown the limitation of classical techniques, particularly for use with very small tissue samples. Accordingly a new enzymatic method for measurement of tissue bicarbonate has been developed.

c. Relationship of ingested glucose to the hepatic synthesis of glycogen:

During the past several years, the concept has emerged that liver is incapable of synthesizing glycogen from ingested glucose without first converting it to lactate and then resynthesizing the three carbon elements to glucose and glycogen. This concept of glucose homeostasis has received wide attention and acceptance and has been called the "glucose paradox." Work in this Laboratory challenges the concept on methodological grounds. Our work suggests rather that after ingestion of oral glucose, a portion of glucose is broken down to give portal vein lactate levels of about 3 mM which undergo gluconeogenesis in liver and conversion to glycogen. A larger portion of ingested glucose is absorbed as glucose, and as portal vein glucose raises to an apparent value of 8 mM, the liver shifts from net glucose output to net glucose uptake into glycogen directly, without first breaking down the absorbed glucose to three carbon precursors. In a related study of glucose metabolism, the thermodynamics of the hydrolysis of sugar phosphate was reinvestigated by members of this Laboratory in response to requests from scientists at the National Bureau of Standards.

d. Measurement of the intermediates of purine and pyrimidine nucleotide biosynthesis and control of the rate of nucleotide synthesis:

Dr. Passonneau, Mr. King and Mr. Teague have been engaged in the development of methods for measurement of intermediates of purine and pyrimidine biosynthesis and interconversion, specifically phosphoribosyl pyrophosphate, and an evaluation of the equilibrium constants of the nucleoside phosphorylase class of reactions and the phosphoribosyl phosphotransferase reactions. This is an attempt to understand the perturbations of nucleotide interconversion and nucleotide synthesis which occur, for example, following ingestion of ethanol, or after initiation of tissue growth with growth factors. A suitable method for measurement of PRPP is expected to be completed before the end of the fiscal year. Purification of adenine phosphoribosyl phosphotransferase has been completed and measurement of its equilibrium constant under physiological concentrations of free [Mg^{2+}] have begun.

Methods for the microdetermination of purine nucleotides and nucleobases using HPLC techniques have been developed for use in the study of cell cultures of fibroblasts of patients with known genetic diseases which obey strict Mendelian laws and whose phenotype can clearly be defined. In collaboration with Dr. Gitomer of the section of Molecular Genetics, analysis of nucleotides from patients with cystic fibrosis suggests that the adenine containing nucleotides are decreased. Associated with this finding is the observation that the Km for adenine phosphoribosyl transferase (EC 2.4.2.7) may be elevated two-fold in the fibroblasts of patients homozygous for cystic fibrosis.

As linkage studies strongly suggest that the primary defect in cystic fibrosis is located on chromosome 7 and adenine phosphoribosyl transferase is located upon chromosome 16, it is unlikely that this defect is primary to the disease of cystic fibrosis. Attempts to measure the phosphorylation potential in cultured fibroblasts and thus relate changes in adenine nucleotide metabolism and phosphorylation potential to Cl^- gradients have yielded values of around 10^6 M^{-1} which are at the absolute limits of energy available in the entire electron transport system. Such values may represent artifacts due to the technical difficulties attendant to the use of cells in culture, but certainly differ markedly from estimates obtained by numerous authors by a variety of techniques in vivo.

Finally, development of other microtechniques for analysis of pertinent metabolites in cultured cells suitable for genetic studies has led to the development of methods for measurement of intermediate and short chain CoA derivatives.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00001-03 LMMB

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 Ethanol on Gastrointestinal Biochemistry and Physiology

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

P.I.:	M.-T. Huang	Chemist	LMMB, NIAAA
Other:	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Metabolism and Metabolic Biology

SECTION

Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.8	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

A surgical method was developed to cannulate chronically both the portal and hepatic veins of laboratory rats. This experimental system is useful for studies on intestinal absorption and hepatic extractions of nutrients. With this experimental system, the following objectives can be determined (1) the effect of ethanol on GI absorption and liver metabolism and (2) glucose paradox. In the first study, the rate of ethanol elimination will be determined in rats meal-fed with diet containing glucose, fructose, mixture of glucose and fructose, and sucrose to determine the importance of alcohol dehydrogenase and redox state in liver on the metabolism of ethanol in vivo. In the second study, portal-hepatic difference of glucose and gluconeogenetic precursors will be determined in order to resolve the paradox that liver can not utilize glucose efficiently. Our results, in the latter subject, show that liver can utilize exogenous glucose and can synthesize glycogen directly from exogenous glucose directly. Recent theory on the pathway of glycogen synthesize in liver (Glucose-C3-G6P-Glucogen) was found to be based on questionable data and inadequate method of calculation.

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

Project Description:

Investigators:

M.T. Huang	Chemist	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA

Objectives:

Accessibility to the portal vein of the common laboratory rat has been a serious experimental obstacle. Because of its location in between gastrointestinal tract and liver, its constituents are not expected to be similar to arterial or venous circulation. For studies on intestinal absorption of nutrients, monitoring changes of concentrations in systemic circulation can produce serious artefact, if liver can sequester the nutrient. We, therefore, decided to develop a chronic cannulation method for the portal vein. The experimental system enables the studies on the intestinal absorption of nutrient in the conscious rats and on the hepatic extraction by measuring portal-hepatic difference of the nutrient.

Two major problems can be studied immediately following the development of the technique. One is on whether liver can utilize glucose. This is pertinent to the so-called "glucose paradox" of Katz and McGarry who concluded that liver can not utilize glucose. Glycogen synthesis in liver is coming from gluconeogenesis, the indirect pathway. The second problem is on the metabolism of ethanol in vivo. It has been suggested that the rate of ethanol metabolism is related to the turnover of cytosolic ATP and redox states of the liver. Cornell, however, concluded that liver alcohol dehydrogenase determines the rate of ethanol metabolism. The rate of alcohol dehydrogenase calculated from a rate equation for a ordered be-be reaction and the kinetic constants of the enzyme coincide with the measured rate of ethanol metabolism. The effect of cytosolic redox state on the metabolism of ethanol is evident only when the intermediates of the malate-aspartate shuttle is artefactually depleted, such as in isolated hepatocytes. Ethanol concentrations in the portal and hepatic vein will be studied in rats meal-fed diet containing different carbohydrates. The intestinal absorption and hepatic metabolism of ethanol can thus be studied.

Methods Employed:

Cannulation: Rats were anesthetized with pentobarbital. Leprotomy was performed with 5 cm longitudinal incision in the abdominal walls. Duodenum was retracted to expose the inferior pancreatoduodenal vein. A PE10 cannula was inserted from pancreatoduodenal vein to the portal vein. The hepatic vein was cannulated through the external jugular vein. The length of the cannula to be inserted was premeasured so that the tip of cannula was placed just below the diaphragm. The cannula were exteriorized in the back of the animal. After the surgery, animals were housed individually.

Meal-feeding: Meal-feeding was started on the second day of the surgery from 9:00 am to 11:00 am for a total period of seven days. Diets were prepared by mixing 50% grounded chow with 50% glucose, 50% fructose, 50% sucrose or 25% glucose + 25% fructose. Body weight and daily intake of diet were recorded. Blood were sampled from the portal and hepatic cannula (0.3 ml each) before and after meal fed on the 1st, 3rd, 5th, and 7th day.

Metabolite assay: Blood plasma was separated from blood cells by centrifugation. Aliquots (20 ul) of plasma were precipitated by sulfosalicylic acid and derivatized with o-phthaldialdehyde in the presence of 3-mercaptopropionic acid. Reverse phase HPLC on C-18

column were used to quantitate the amino acid content of the plasma. The concentration of glucose, fructose, lactate, and pyruvate were measured enzymatically from another aliquot of plasma (0.1 ml) prepared by 3.6% PCA and neutralization.

The metabolism of ethanol: On the seventh day after the end of meal feeding, the rats were briefly anesthetized with ether. A dose of ethanol (5 u; 1 ml/100 g 6.w) was intubated. Blood was sampled through the portal and hepatic vein at 1, 5, 20, and 40 min after the intubation. Ethanol was assayed enzymatically with alcohol dehydrogenase.

Major Findings:

Rats: Effects of surgery on body wt and food intake were followed for a period of seven days. Body weight decrease substantially (8%) on the first day post-surgically. Thereafter, the rate of decrease of body weight alleviated. On the third or fourth day after the surgery, body weight of the animal stabilized. A slight increase in body weight was observed on the sixth or seventh days after the surgery. No food intake was observed on the first and second day after the surgery. On the third day, the amount of diet consumed was 5 g, regardless of the composition of the diet. From the fourth day on, daily food intake increased to above 10 g per day.

Hepatic-portal difference of glucose concentration: Glucose concentration in plasma of portal and hepatic blood was determined. It was found that in fasted state liver generally released glucose as the glucose concentration in the hepatic vein was found to be higher than that in the portal vein. After meal, hepatic-portal difference of glucose concentration seems to correlate with the glucose concentration in the portal vein, with crossover point at a portal concentration of 8 mM. Above this level, net extraction of glucose can be seen in fructose- and glucose-supplemental rats. Thus, it seems that liver can utilize glucose, but only when the concentration in the portal vein is raised above 8 mM. The significance of this finding to "glucose paradox" has to be studied further. The hepatic extraction can be converted to the rate of glucose utilization if the rate of blood flow in liver is known.

Metabolism of ethanol in vivo: In one study with a glucose-fed and fructose-fed rat, the rate of disappearance of orally ingested ethanol in hepatic blood are not significantly different. However, the concentration of ethanol in the two rats were not the same. Further studies are necessary to determine the effect of dietary carbohydrate on the absorption of ethanol and liver extraction of ethanol.

Significance to Biomedical Research and the Program of the Institute:

Present work describe a method to open up the portal vein of laboratory rat for blood sampling and injection. Studies involving this experimental system have been initiated to resolve the issue of glucose paradox, whether liver can utilize glucose or the effect of fructose on the metabolism of ethanol.

Proposed Course:

In the coming year, the effect of ethanol on the biochemistry and physiology of GI will be studied.

Publications:

Huang MT, Veech, RL. Role of the direct and indirect pathways for glycogen synthesis in rat liver in the postprandial state, J Clin Invest 1988;81:872-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00035-02 LMMB

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 Ethanol and its Metabolites on Metabolism and Inorganic Ion Balance

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

P.I.:	W.L. Gitomer	Chemist	LMMB, NIAAA
Others:	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS (if any)

R.L. Ornberg, LCBG, NIDDK; R.D. Leapman, BEI, DRS

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Ave., Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

It was observed that the treatment of 48hr. starved rats with acetate, propionate or butyrate results in large increases in the hepatic Ca2+, Mg2+ and inorganic pyrophosphate (PPi) content apparantly due to the formation of calcium and magnesium PPi precipitates within the mitochondrial matrix. The increase in mitochondrial calcium and magnesium was shown to occur using electron probe x-ray microanalysis. Assuming that the free matrix [Mg2+]=1 mM and using the magnesium PPi and calcium PPi solubility products, the free mitochondrial [Ca2+] in the liver was calculated to be 1.2 mM after treatment with short chain fatty acids. This observation was then expanded to all metabolic states and it was concluded that under all in vivo conditions thus far studied that, in the in vivo rat liver, calcium and magnesium PPi precipitates are present in the mitochondrial matrix and the free mitochondrial matrix [Ca2+] is about 1mM. This value is three orders of magnitude greater than values estimated for the free mitochondrial matrix [Ca2+] using isolated mitochondria.

PHS 6040 (Rev. 1/84)

Project Description:

Investigators:

W.L. Gitomer	Chemist	LMMB,NIAAA
R.L. Veech	Chief	LMMB,NIAAA
R.L. Ornberg		LCBG,NIDDK
R.D. Leapman		BEI,DRS

Ojectives:

Ethanol is metabolized in the liver by alcohlol dehydrodgenase to acetaldhyde and acetaldhyde is further metabolized by aldehyde dehydrogenase to acetate. Most emphasis in the study of the metabolities of ethanol has focused on acetaldhyde. However, the blood concentration of this metabolite rarely exceeds 10uM, while the blood concentration of acetate approaches 2mM even after the consumption of moderate amounts of ethanol. This study was undertaken to elucidate the consequences of acetate metabolism on hepatic inorganic ion content.

Methods Employed:

1. Atomic absorption spectrophtometry for the measurement of inorganic cations.

2. Electron probe x-ray microanalysis for the subcelluar localization of inorganic elements.

3. The incubation of isolated hepatocytes under the conditions necessary for the cells to accumulate PPi like the in vivo liver.

4. Enzymatic determination of PPi

5. Dignition fractionation of isolated hepatocytes.

Major Findings:

It has been shown that the treatment of 48hr. starved rats with acetate, propionate or butyrate results in large increases in the hepatic Ca^{2+}, Mg^{2+} and inorganic pyrophosphate [PPi] content apparently due to the formation of calcium and magnesium PPi precipitates within the mitochondrial matrix. From these observations it has been deduced that (1) the free mitochondrial [Ca^{2+}] in the liver is 1.2 mM under all in vivo conditions thus far studied, (2) in the in vivo rat liver calcium and magnesium PPi precipitates are present in the mitochondrial matrix, (3) the free energy of the calcium concentration gradient between the mitochondria and the cystol is equal to the free energy of the cystolic phosphorylation potential, (4) the gradient of free calcium between the mitochondria and the cystol is either in near-equilibrium with the mitochondrial membrane potential of -123 mV or the free calcium concentration gradient is the in vivo energy transducer for oxidative phosphorylation. (5) calcium phosphate and carbonate precipitates are likely to occur in the mitochondrial matrix and the physical properties of these inorganic salts set maximum possible values for both the pH within the mitochondria and the the pH gradient between the mitochondrial and the cystol, (6) that the apparent disagreement between the chemical measurements of phosphate in tissue and the measurement of phosphate by NMR is due to the sequestration of phosphate with in the mitochondria, and (7) that the concentration of free cytosolic ADP does not regulate the rate of oxygen consumption under most in vivo conditions as proposed by Chance and Williams but that it is regulated by the free cytosolic calcium concentration.

Significance to Biomedical Research and the Program of the Institute

Elevation of intracellular PPi causes some of the largest changes in cellular calcium content yet observed. Intracellular Ca^{2+} is one of the major cellular second messengers and in this role, it acts as a regulator of a multitude of metabolic reactions. The role of PPi in regulating cellular Ca^{2+} will now have to be considered.

It has been proposed that the activity of the Tricarboxycylic Acid Cycle is regulated by micromolar concentrations of Ca^{2+} in the matrix of the mitochondria. The finding that the mitochondrial Ca^{2+} concentration is 1mM means that the activity of the enzymes in vivo is not regulated by Ca^{2+}.

Finally a number of disease states found in alcoholics are related to changes in calcium content of tissues including bone disease. This disease state should now be considered from the point of view of the effects of acetate metabolism on tissue inorganic ion content.

Proposed Course:

Acetate is the major metabolic product of ethanol metabolism in the liver with about 90% of the acetate which is produced during ethanol metabolism and is released from the liver into the general blood supply. This large "export" of acetate from the liver would be expected to have a profound effect on the inorganic ion balance of a number of different tissues. In the liver, it would be expected to have the opposite effect on ion balance that has been observed on the uptake and metabolism of acetate. While in acetate utilizing tissues, such as heart, changes in inorganic ion balance would be expected to be similar to the acetate treated liver. Thus, the effect of ethanol metabolism on the inorganic ion balance of liver, blood and other tissues will be examined.

Publications:

Gitomer WL, Veech RL. Application of near-equilibrium themodynamics to living systems and the estimation of free mitochondrial Ca^{2+} concentration. In: Lemasters JL, Hackenbrock CR, Thurman RG, Westerhoff HV eds. Integration of Mitochondrial Function. New York: Plenum Publishing Corporation, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00023-10 LMMB

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 Ethanol on Metabolic Control Processes

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

P.I.:	R.L. Veech	Chief	LMMB, NIAAA
Others:	W.L. Gitomer	Chemist	LMMB, NIAAA

COOPERATING UNITS (if any)

LCBG, NIDDK (R. Ornberg)

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Ethanol is metabolized in the liver by alcohol dehydrogenase to acetaldehyde, and acetaldehyde is then further metabolized by aldehyde dehydrogenase to acetate. Most emphasis in the study of the metabolites of ethanol has focused on acetaldehyde. However, the blood concentration of this compound rarely exceeds 10 uM, while the blood concentration of acetate approaches 2 mM even after the consumption of moderate amounts of ethanol. This study was undertaken to elucidate the consequences of acetate metabolism on the major metabolic pathways.

Administration of acetate to starved rats causes a number of changes in hepatic metabolite concentrations, the most striking of which are (1) a greater than one hundredfold elevation in pyrophosphate concentration, (2) a fivefold elevation in calcium, (3) a marked reduction in the cytosolic [NADP-+]/[NADPH] redox ratio,, and (4) a twofold increase in glucose concentration.

This project has been terminated.

Gitomer, WL, Veech RL. The determination of the in vivo mitochondrial free Ca^{2+} concentration. In: Leemaster J, ed. Integration of Mitchondrial function. Plenum Press: New York. in press.

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00024-10 LMMB

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 and Metabolic Studies of Human Alcoholics

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

P.I.:	R.L. Veech	Chief	LMMB, NIAAA
Others:	J.P. Casazza	Chemist	LMMB, NIAAA

COOPERATING UNITS (if any)

Department of Academic Medicine, London, England

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Metabolic Control

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.6	0.6

CHECK APPROPRIATE BOX(ES)

[X] (a) Human subjects [X] (b) Human tissues [X] (c) Neither

[ ] (a1) Minors

[X] (a2) Interviews

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

In three separate studies involving three different sets of collaborators, elevated levels of 2,3-butanediol have been found in the blood of 80% of chronic alcoholics, but not social drinkers consuming distilled spirits. Two separate methods of gas chromatographic analysis of diols have been developed. One method involving formation of the bromophenylboronate derivative can accurately measure to d-1, or meso 2,3-butanediol to 5 uM.

In the rat, two pathways of butanediol formation have been demonstrated. The first (Veech RL, et al. Curr Top Cell Regul 1981;18:151-179) involves elevated blood acetaldehyde entering the brain with an active pyruvate dehydrogenase multi-enzyme complex where it condenses with hydroxyethyl thiamine pyrophosphate to form acetoin. The acetoin is subsequently converted in liver to 2,3-butanediol. In a second animal model, 2,3-butanediol in the rat is produced by acetone feeding. Prolonged fasting in man, however, produces diols but not 2,3-butanediol, suggesting differences in the metabolic pathways between man and the rat. Whether 2,3-butanediol in blood may be used as a "genetic marker" for alcoholism or is an induced defect can only be determined by a controlled study involving alcohol administration under controlled conditions on a metabolic ward.

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

Project Description:

Investigators:

R.L. Veech	Chief	LMMB, NIAAA
J.P. Casazza	Chemist	LMMB, NIAAA
E.D. Teague	Biologist	LMMB, NIAAA
M. Morgan	Professor	Dept. Academic Medicine, London, England

Objectives:

The purpose of this series of studies was to determine an aberrant metabolic pathway results in the producing of 1,2-propanediol and 2,3-butanediol in alcoholics. The hypothesis to be tested thus derives from the classical medical paradigm and seeks to define a certain subset of people with alcohol problems who may be classified as being addicted to alcohol or are alcoholics (Rutstein D, and Veech RL: N Engl J Med 1978;298:1140-1141). The hypothesis to be tested, therefore, differs from the view that alcoholism is the result of deviant behavior patterns rooted in the sociocultural patterns of alcohol consumption and other forms of substance abuse.

Once abnormal or unusual metabolic consequences were identified within the alcoholic population, it followed that (1) accurate methods for measuring these compounds needed to be developed, and (2) the aberrant enzyme or gene product responsible for the metabolic difference needed identification. With accomplishment of these goals it would be possible, given an appropriate setting, to achieve a rational biochemical basis for the diagnosis of alcohol addiction by objective laboratory means and to determine whether these findings are a cause or a result of the alcoholism.

Methods Employed:

Identification of the abnormal metabolites was accomplished on contract (Borrison and JTC) using GC mass spectrometry. Measurement of diol was accomplished by use of a Varian gas chromatograph with electron capture detection. Acetol was measured as the dinitrophenylhydrazone derivative by HPLC. Synthesis of chemical intermediates of the diol pathway was accomplished by standard techniques of organic chemistry.

Major Findings:

Clinical Studies: Previous studies have shown blood 2,3-butanediol in 70% of blood samples and blood 1,2-propanediol in 90% of all samples drawn from alcoholics without liver disease when intoxicated (Rutstein DD, Veech RL, Nickerson RJ, Felver ME, Vernon AA, Needham LL, Kishore P, Thacker SB. Lancet 1983;ii:534-536). In alcoholics without liver disease as serum ethanol decreased so did the level of 2,3-butanediol. In most cases 2,3-butanediol was not measurable 32 hours after admission to a detoxification center (Veech RL, Felver ME, Lakshmanan MR, Huang M-T, Wolf S. In: Estabrook R, Srere P eds. Current topic in cellular regulation. New York: Academic Press 1981;17:151-179). No 2,3-butanediol was measurable in control subjects in the absence of ethanol. In a more recent study (Casazza JP, Stambuk D, Frietas J, Morgan MY, Veech RL., manuscript in preparation), ethanol-free blood samples were drawn from 53 normal reference subjects, 45 patients with alcoholic fatty liver, 10 patients with

alcoholic hepatitis, 50 patients with alcoholic cirrhosis and 77 patients with non-alcoholic liver disease. 1,2-Propanediol was found in 8% of the reference samples, 15% of the patients with alcoholic fatty livers, 40% of the patients with alcoholic hepatitis, 46% of the patients with alcoholic cirrhosis and 49% of the patients with non-alcoholic liver disease. These data indicate that in the absence of ethanol, 1,2-propanediol is an indicator of general liver disease. Furthermore, administration of ethanol to controls and patients with non-alcoholic liver disease with measurable 1,2-propanediol resulted in a significant increase in 1,2-propanediol levels. Based on these data, it seems likely that the high incidence of 1,2-propanediol, previously found in blood drawn from alcoholics who were drinking distilled spirits (Rutstein et al. Lancet 1983;ii:534-536), was the result of ethanol raising 1,2-propanediol levels in a population capable of producing 1,2-propanediol due to pre-existing liver disease.

D,L-2,3-Butanediol was a more specific marker of alcoholic disease. D,L-2,3-butanediol was present in ethanol-free blood samples from 2% of the normal reference group, 9% of the patients with alcoholic fatty liver, 26% of the patients with alcoholic cirrhosis and 3% of the patients with non-alcoholic liver disease. The incidence of this compound within each group was independent of commonly used blood tests for liver disease. Administration of distilled spirits to 32 control subjects and 10 patients with non-alcoholic liver disease under controlled conditions did not result in any measurable D,L-2,3-butanediol. There was no difference in the incidence of D,L-2,3-butanediol in patients with alcoholic cirrhosis between those that had been ethanol abstinent two weeks or more and those that had not. In fact, the highest level of D,L-2,3-butanediol was identified in a patient who had been ethanol abstinent for 5 years. Clearly, despite the association of D,L-2,3-butanediol with alcoholism, the production of D,L-2,3-butanediol in these patients is not directly related to either ethanol metabolism or its metabolic products.

Metabolic Studies:

An ethanol inducible pathway for the production of 1,2-propanediol has been described in rat (Casazza JP, Felver ME, Veech RL. J Biol Chem 1984;259:231-236). The major inducible enzyme in this pathway has been shown to be P-450 2e, the major ethanol inducible enzyme (Koop DE, Casazza JP. J Biol Chem 1985;260:13607-13612). This enzyme has now been cloned (Song BJ, Gelboin HV, Park SS, Yang CS, Gonzalez FJ. J Biol Chem 1986;261:16687-16697). It has been suggested that P-450 2e plays a role in human oncogenesis (Thomas PE, Bandiera S, Maines SL, Ryan DE, Levin W. Biochemistry 1987;26:2280-2289). Clinical evidence of 1,2-propanediol production now indicates that this enzyme may play a role in liver disease.

Significance to Biomedical Research and the Program of the Institute:

The specificity of D,L-2,3-butanediol to alcoholic disease makes this compound of particular interest to alcohol research. The data to date are still compatible with but not conclusive of the possibility that the enzymes responsible for the elevation of D,L-2,3-butanediol represent an inherited metabolic abnormality distinguishing alcoholics from nonalcoholics. Even if D,L-2,3-butanediol is not a genetic indicator for alcoholism, a metabolic indicator of excessive alcohol consumption would be invaluable as a diagnostic tool for alcoholism. The close correlation between the incidence of 1,2-propanediol and liver disease suggests that understanding the mechanism of production of this compound may help explain the causes of liver disease.

Proposed Course:

Work in the Laboratory of Metabolism and Molecular Biology has identified D,L-2,3-butanediol as a specific indicator of alcoholic disease in both the absence of and the presence of ethanol. New animal models have been developed which result in the production of D,L-2,3-butanediol. In collaboration with the Royal Free Hospital, further studies into the production of 1,2-propanediol, meso-2,3-butanediol and D,L-2,3-butanediol are currently in progress. Known diol producers are being given postulated metabolic precursors in an attempt to further define the means of production of these compounds in humans. Work in this laboratory has led to the identification of 1,2-propanediol as a marker of liver disease, the description of a pathway for its production, and the cloning of the primary inducible enzyme of this pathway. Both the analytical methods developed for metabolic analysis and the techniques of molecular which have been developed are now being used to assess the role of 1,2-propanediol production in liver disease. Further studies into the mechanism of production of this compound in humans and the role of genetics in the production of these compounds, using the analytical techniques developed for metabolic analysis and the techniques of molecular biology, should give an insight into the etiology of alcoholism and liver disease.

Publications:

1. Casazza JP, Frietas J, Stambuk D, Morgan MY, Veech RL. The measurement of 1,2-propanediol, D,L-2,3-butanediol and meso-2,3-butanediol in controls and alcoholic cirrhotics. Alcohol and Alcoholism 1987;(suppl 1):607-609.

2. Casazza JP, and Veech RL. The production of 1,2-propanediol and 2,3-butanediol in severe alcoholics in Human Metabolism of Alcohol. In: Crow K, ed. Boca Raton, CRC Press. in press.

3. Casazza JP, Frietas J, Stambuk D, Morgan M, Veech RL. The measurement of D,L-2,3-butanediol in controls and patients with alcoholic cirrhosis. Advances in Alcohol and Substance Abuse. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00034-04 LMMB

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 the Level of Pentose Cycle Intermediates In Vivo.

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

P.I.:	J.P. Casazza	Chemist	LMMB, NIAAA
Others:	R.L. Veech	Chief	LMMB, NIAAA
	W.T. Schaffer	Executive Secretary	OFA, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Metabolism and Molecular Biology

SECTION
Molecular Genetics

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

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

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

Of the pathological conditions associated with alcoholism, the etiology of Wernicke-Korsakoff's syndrome is one of the most well defined. Thiamine deficiency is clearly established as the causal factor. The pentose cycle, which supplies NADPH for the maintenance of cellular integrity and for fatty acid synthesis, is dependent on transketolase, a thiamine-dependent enzyme, for the functioning of this pathway. Despite the importance of the pentose cycle, neither control of flux nor of the level of pentose cycle intermediates is well understood. We have shown that in both starved and ad libitum fed animals the level of pentose cycle metabolites and metabolites of glycolysis are interdependent. The equilibrium relationships defined by the enzymes of the nonoxidative pentose cycle result in considerable stability in the level of pentose cycle intermediates. In dietary situations where the relative amounts of transketolase and transaldolase decrease with respect to glucose 6-P dehydrogenase, these equilibrium relationships are not valid. Under these circumstances, the tissue content of pentose cycle intermediates are elevated more than twenty-fold above those observed in starved animals.

This project has been terminated.

PHS 6040 (Rev. 1/84)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00019-09 LMMB

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.)*

Pyrazoles as Affectors of Alcohol Dehydrogenase and Cytochrome P-450.

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

P.I.:	N.W. Cornell	Research Chemist	LMMB, NIAAA
Others:	P.W. Wiesenfeld	Guest Scientist	LMMB, NIAAA
	M.V. McGuire	Biologist	LMMB, NIAAA

COOPERATING UNITS *(if any)*

Biochemistry Department, Dartmouth University, Hanover, NH (J. Sinclair). Biochemistry Department, Michigan Medical School, Ann Arbor, MI (D. Koop).

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.50	1.25	0.25

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

All of these studies involve quantitative structure-activity analyses of pyrazole derivatives as probes of enzyme catalytic sites in vitro and as effectors of those enzymes within whole cells. This work began as an attempt to learn whether the properties of alcohol dehydrogenase determined with liver extracts have predictive value for that enzyme functioning in ethanol metabolism in vivo. The conclusions of that initial study had implications not only about alcohol dehydrogenase but also about the relative unimportance of other pathways of ethanol oxidation. However, those conclusions were potentially compromised by the demonstration in another laboratory that pyrazoles do, in contrast to long-standing assertions, inhibit microsomal ethanol oxidation. Thus, it became necessary to determine the structure-activity relationship for pyrazoles acting as ligands for cytochrome P450. That determination, in turn, suggested experiments with pyrazoles as inducers of P450 and the results of these experiments have provoked a working hypothesis about the unexplained mechanism of induction of some types of P450.

This project has been terminated.

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

Publications:

1. Cornell NW, Sinclair JF, Stegeman JJ, Hansch C. Pyrazoles as effectors of ethanol oxidizing enzymes and inducers of cytochrome P450. Alcohol and Alcoholism 1987; (suppl 1): 251-255.

2. Hayes, AL, Marden LJ, McGuire MV, Cornell NW. Induction of rat hepatic drug metabolizing enzymes by parazole. Advances in Alcohol and Substance Abuse. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00026-06 LMMB

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.)

Subcellular Distribution of Enzymes

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

P.I.:	N.W. Cornell	Research Chemist	LMMB, NIAAA
Others:	P.W. Wiesenfeld	Guest Scientist	LMMB, NIAAA
	M.V. McGuire	Biologist	LMMB, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The metabolism of ethanol perturbs the major nucleotides of liver, the pyridine nucleotides (NAD, NADH), and the adenine nucleotides (ATP, ADP, AMP). Distributions of adenine nucleotides are also central to considerations of cellular bioenergetics, and accurate quantitative data for the subcellular distributions of enzymes are essential in analyzing metabolism in vivo. Compared with traditional techniques of tissue homogenization, digitonin fractionation of isolated hepatocytes yields a much faster and, in some cases, more accurate determination of enzyme compartmentation. Results with ATP citrate lyase are illustrative. Although previously thought to be entirely cytosolic, digitonin fractionation shows that a portion of total cellular ATP citrate lyase is bound to mitochondria or some other structure. The amount bound varies with the animal's nutritional state. In hepatocytes from rats that were either starved 2 days and then fed NIH stock diet ad libitum, or starved 2 days and then refed a fat-free diet 2 days, the noncytosolic activity was, respectively, 52%, 21%, or 24% of total cellular lyase. Because starvation/refeeding strongly induces lipogenic enzymes, the amount of bound lyase activity in this dietary state was 10 to 12 times greater than in rats that were starved or fed ad libitum. The association of citrate lyase with a subcellular organelle is also influenced by CoA. Adding 20 uM CoA to the digitonin fractionation medium caused all the lyase to be released from cells like a cytosolic enzyme. Conversely, when cellular free CoA was decreased by incubating hepatocytes with the hypolipidemic agent, 5-(tetradecyloxy)-2-furoic acid, the amount of bound lyase was increased. These results suggest that the noncytosolic ATP citrate lyase may have a special role in lipogenesis. Intracellular compartmentation and metabolic zonation can also be studied by perfusing the intact liver with digitonin, and calcium ions have a strong influence on relative patterns of enzyme release from the cytosol and mitochondria. This procedure has led to ways of isolating periportal and perivenous hepatocytes, enabling the study in vitro of metabolic zonation.
This project has been terminated.

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00027-06 LMMB

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.)*

Induction of Aminolevulinic Acid Synthase in Hepatocytes

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

P.I.:	N.W. Cornell	Research Chemist	LMMB, NIAAA
Others:	P.W. Wiesenfeld	Guest Scientist	LMMB, NIAAA
	M.V. McGuire	Biologist	

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The ingestion of alcohol causes porphyria in man and, with it, an increase in aminolevulinic acid synthase, the initial enzyme of heme biosynthesis. Since that enzyme is synthesized in the cytosol under the control of the nuclear genome, but must move from the cytosol into the mitochondria to function in heme biosynthesis, there are obviously many steps at which ethanol might act to increase its cellular activity. Previous studies in this laboratory have shown that the digitonin fractionation of isolated hepatocytes is an unusually good method for obtaining rapid information about the subcellular distribution of macromolecules, and this project was initiated to apply that method to analyze the induction of the synthase and its movement between cytosol and mitochondria. Evidence has been presented that, in chick embryo liver, the enzyme appears in the cytosol initially as a 74,000-molecular weight precursor that is converted to a 68,000-molecular weight protein during transit into the mitochondria. In contrast, for rat liver, it has been reported that both the cytosolic and mitochondrial ALAS proteins are dimers of 51,000-molecular weight subunits. These discrepancies may really reflect species differences, or they may simply arise from technical difficulties in extracting the native protein. Another contrast between the chick embryo and rat, mouse, guinea pig, as well as the adult chicken, is that all of the other named species can acquire substantial levels of ALAS in the cytosol following treatment with inducers (see below), whereas no cytosolic ALAS occurs in the chick embryo liver. These problems are being pursued by establishing conditions for the induction of aminolevulinic acid synthase in isolated liver cells and fractionation of these cells with digitonin. A cDNA probe is being developed to permit quantitation of ALAS mRNA levels in response to nutritional, alcohol and xenobiotic treatments.

This project has been terminated.

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

Publications:

King MT, Reiss PD, Cornell NW. Determination of short chain coenzyme-A compounds by reversed phase high-performance liquid chromatography, Meth Enzymol. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00033-05 LMMB

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.)

Metabolic Effects of Growth Factors and Growth Hormone

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

P.I.:	B.Y. Reed	Senior Staff Fellow	LMMB, NIAAA
Others:	M.J. Gerhart	Chemist	LMMB, NIAAA
	M.T. King	Chemist	LMMB, NIAAA
	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	1.1	1.0

CHECK APPROPRIATE BOX(ES)

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

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

An important effect of ethanol is to disrupt cellular growth. Specifically ethanol has been shown to inhibit hepatocyte DNA synthesis by a number of agents including epidermal growth factor (EGF)(Carter EA, Wands JR, Biochem Biophys Res Commun 1985;128:767-774). In an attempt to understand the mechanism by which ethanol interferes with the normal processes of growth and development we studied the early metabolite changes induced by EGF, platelet derived growth factor (PDGF) and angiotensin in rat liver in vivo (Reed BY, King MT, Gitomer WL, Veech RL, J Biol Chem 1987;262:8712-8715; Reed BY, King MT, Gerhart MJ, Veech RL, Biochem Soc Trans 1988;16:636-637). Elucidation of the metabolic changes induced enabled us to identify 2 enzymes affected by the actions of EGF and PDGF respectively. We have subsequently demonstrated a direct effect of ethanol on the normal metabolic action of EGF in vivo (Gerhart MJ, Reed BY, Veech RL, Alcoholism: Clin and Exp Res 1988;12:116-118) and further shown that the apparent modulation of the action of EGF by ethanol occurs at an intracellular site as ethanol does not interfere with the binding of the growth factor to its receptor (Gerhart MJ, Reed BY, Veech RL, In: Advances in Alcohol and Substance Abuse. 1988; in press). Currently further studies are in progress to elucidate the role of PDGF in alcoholic liver disease.

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

Project Description:

Investigators:

B.Y. Reed	Senior Staff Fellow	LMMB, NIAAA
M.J. Gerhart	Chemist	LMMB, NIAAA
M.T. King	Chemist	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA

Objectives:

The aims of this project are to investigate the cellular mode of action of growth factors and to assess the contribution made by growth factor action to alcoholic liver damage and normal growth and development.

Methods Employed:

Rats are injected with an appropriate dose of the growth factor or hormone in question. The hormone treatment in the case of the ethanol studies is preceeded by an acute dose of ethanol. The animals are sacrificed 5 min after administration of the hormone and metabolite levels and enzyme activities measured by established techniques on samples of their freeze clamped liver. All hormone treated animals are compared with similar data collected from saline injected controls.

Major Findings:

EGF, unlike PDGF, appears to act at 2 discrete site in the glycolytic pathway. While identification of an activity change in the enzyme pyruvate kinase [EC 2.7.1.40] was readily demonstrable no enzyme change was found to explain the apparent change in glucose and glucose 6-P contents. As the enzymes which control the level of these metabolites have been reported to undergo complex regulation via translocation we re-examined these enzymes looking for any change in their activity in various cellular fractions. No change was seen suggesting that either under the in vitro assay condition the change in activity is no longer expressed or regulation of these metabolites is by an as yet unknown path.

As we successfully identified a kinetic change on the activities of glucose 6-P dehydrogenase (PDGF) and pyruvate kinase (EGF) we wished to identify the nature of the protein modification in each case. Many proteins are modified in a reversible manner necessitating a rapid isolation technique for the protein under study. We have prepared appropriate antibodies directed against these enzymes to facilitate this need. The initial finding that ethanol modulated certain metabolic effects of EGF in vivo prompted us to further examine the mechanism by which this occurred. The most obvious site of action would be at the receptor however, we have shown that the binding of EGF to its receptor is normal in the presence of ethanol. Indicating that the action of ethanol is most likely at an intracellular level.

Significance to Biomedical Research and the Program of the Institute:

One of the important effects of ethanol is the disordered regrowth of hepatic cells which is characteristic of alcoholic hepatitis and cirrhosis. Multiple growth factors play a role in normal cellular division and growth in liver and other tissues. It is important to understand of the basic mechanism by which growth factors act at a cellular level and gain insight into some of the medical complications of alcoholism.

Proposed Course:

We are currently utilizing the antibodies prepared against the enzymes of interest in the action of EGF and PDGF to identify the nature of the protein modification in each case. As PDGF is known to play a key role in wound healing and cellular division investigation of the interaction between PDGF and ethanol is planned in an in vivo system and a tissue culture milieu.

Publications:

Gerhart MJ, Reed BY, Veech RL. Ethanol inhibits some of the early effects of epidermal growth factor in vivo. Alcoholism: Clinical and Experimental Research 1988;12(suppl 1):116-118.

Reed BY, King MT, Gerhart MJ, Veech RL. Effects of angiotensin in the liver of the rat in vivo, Biochem Soc Trans 1988;16:636-637.

Gerhart MJ, Reed BY, Veech RL. Epidermal growth factor binding in the presence of ethanol. In: Advances in Alcohol and Substance Abuse. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00036-02 LMMB

PERIOD COVERED

June 1, 1987 to September 30, 1988

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

Structure and regulation of ethanol-inducible cytochrome P450 gene

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

P.I.:	B.J. Song	Senior Staff Fellow	LMMB, NIAAA
Others:	R.L. Veech	Chief	LMMB, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Molecular Carcinogenesis, National Cancer Institute (F.J. Gonzalez)

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The microsomal enzyme induced by feeding of alcohol is also the same enzyme responsible for the elevation of serum 1,2-propanediol found in the serum of alcoholics (Koop DR, Casazza JP, J Biol Chem 1985;260:13607-13612). We have cloned rat and human cDNA encoding ethanol-inducible cytochrome P450 (P450 IIE1) and determined their nucleotide and thus protein sequences. Using cloned P450 IIE1 cDNA and antibodies, the regulation of P450 IIE1 gene expression was examined. We have proposed three distinct types of regulation of P450 IIE1 gene expression: transcriptional activation during development; post-transcriptional activation (probably via protein stabilization) by various inducers such as ethanol, acetone, and pyrazole derivatives; and mRNA stabilization in diabetes and starved animals. By cloning and sequencing of rat genomic DNA for P450 IIE1, we have demonstrated evidences that the transcriptional activation of P450 IIE1 is due to specific demethylation only at 5' end of the P450 IIE1 gene. By measuring the turnover rates of P450 IIE1 from untreated control rats and acetone-treated rats, we further suggest that post-transcriptional activation by various exogeneous inducers are due to specific P450 IIE1 protein stabilization.

PHS 6040 (Rev. 1/84)

Project Description:

Investigators:

B.J. Song	Senior Staff Fellow	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA
F.J. Gonzalez	Acting Section Chief	LMC, NCI, NIH

Objectives:

The structure of P450 IIE1 gene was to be determined and studied for the sites of transcriptional activation during early development. The mechanism of post-transcriptional activation of P450 IIE1 by various inducers such as ethanol, acetone and pyrazole derivatives was further studied. In order to correlate the levels of propanediol in human subjects and P450 IIE1, the development of specific method for detecting P450 IIE1 in human tissues than are easily obtainable such as lymphocytes was initiated.

Methods Employed:

P450 IIE1 gene was cloned from rat genomic DNA library by hybridization technique using specific cDNA for P450 IIE1. Its whole nucleotide sequence was determined by dideoxy chain termination method. The regulation in the 5' and 3' ends of P450 IIE1 gene was carefully examined during the early development especially between neonate and 1 week old rats. For the mechanism of post-transcriptional activation, the turnover rates of P450 IIE1 were estimated for the untreated and acetone-treated rats. After labeling with [^{14}C]bicarbonate and [^{3}H]leucine, for different times, P450 II from each group at various times was purified by monoclonal antibody immunoaffinity columns. The immunopurified P450 II#1 was either subjected to autoradiography or to direct liquid scintillation counting.

Major Findings:

(1) The whole nucleotide sequence coding for P450 IIE1 gene has been determined.

(2) The post-transcriptional activation during early development is related to specific demethylation of the 5' and of the P450 IIE1 gene.

(3) The turnover rates of P450 IIE1 in untreated control rats demonstrated a typical biphasic pattern with half-lives of 7 h and 37 h, respectively.

(4) However, in acetone-treated rats, only the slower one with a half-life 37 h was observed which indicates that the elevation of P450 IIE1 by acetone is mainly due to P450 IIE1 protein stabilization.

(5) P450 IIE1 can be detected by immunoblot analyses in human lymphocytes and its level was elevated in poorly-controlled juvenile diabetics than the age-matched control groups.

Significance to Biomedical Research and the Program of the Institute:

Chronic intake of ethanol in both animal and human subjects leads to increases in the production of 1,2-propanediol, one of two unusual metabolites often found in human alcoholics blood (Rutstein DD, et al., Lancet 1983;ii:534-537). The P450 IIE1 has been

demonstrated to be the responsible enzyme for the production of propanediol in experimental animals (Koop DR, Casazza JP, J Biol Chem 1985;260:13607-13612). The works of basic protein biochemistry and future studies on human P450 IIE1 from human specimens as analyzed by restriction fragment length polymorphism along with immunochemical analysis would give insights why and how people have different levels of propanediol after drinking alcohol.

Proposed Course:

Work will continue on the development of detection method for P450 IIE1 in human specimens that are easily obtainable.

Publications:

Song BJ, Matsunaga T, Hardwick JW, Veech RL, Yang CS, Gelboin HV, Gonzalez FJ. Stabilization of cytochrome P450j mRNA in the diabetic rat, Mol Endocrinol 1987;1:542-547.

Umeno M, Song BJ, Kozak C, Gelboin HV, Gonzalez FJ. The rat P450 IIE1 gene: complete intion and exon sequence, chromosomal mapping, and correlation of developmental expression with specific 5' cytosine demethylation, J Biol Chem 1988;263:4956-4962.

Song BJ, Veech RL, Park SS, Gelboin HV, Gonzalez FJ. Structure and regulation of the ethanol-inducible cytochrome P450j, In: Stimmel, eds, Advances in Alcohol and Substance Abuse. New York: Academic Press 1988, in press.

Song BJ, Hardwick JP, Gelboin HV, Gonzalez FJ. Structure of ethanol-inducible cytochrome P450 and three different types of regulation in P450j expression. The Second International Workshop on P450 Gene Regulation, 1987.

Song BJ, Veech RL, Park SS, Gelboin HV, Gonzalez FJ. Cytochrome P450 IIE1 (P450j) protein stabilization by acetone. The 72nd annual FASEB meeting, Las Vegas, 1987.

Saenger P, Veech RL, Song BJ. Increased cytochrome P450j (P450j) levels in lymphocytes of children with poorly controlled insulin dependent diabetes mellitus (IDDM). Annual meeting of Society for Pediatric Research, Washington D.C. 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00037-02 LMMB

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 cloning of pyruvate dehydrogenase gene

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

P.I.	B.J. Song	Senior Staff Fellow	LMMB, NIAAA
Others:	R.L. Veech	Chief	LMMB, NIAAA
	T.L. Huh	Visiting Fellow	LMMB, NIAAA
	Y.T. Chi	Visiting Fellow	LMMB, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Molecular Genetics

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

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

Recent studies from our laboratory indicate that 2,3-butanediol, one of two unusual metabolites found in human alcoholic blood, is predominantly associated with human subjects who suffer alcoholic hepatitis or alcoholic cirrhosis (Casazza, et al., Alcohol and Alcoholism 1987;(suppl 1):607-609). Although the exact mechanism of 2,3-butanediol formation is not known, Veech and his associates have postulated that it could be generated by pyruvate dehydrogenase that are found in brain and testis (Curr Top Cell Regulation 1981;18:151-179). Based on the proposal, we have just started a project to clone genes encoding for pyruvate dehydrogenase complex using synthetic oligodexoynucleotide probes. By nucleotide sequencing, we have confirmed that our clones for E_{1x} and E_{1B} and E_3 represent the authentic pyruvate dehydrogenase. Using these cDNA clones, we are studying the regulation of these genes especially in alcoholic subjects.

PHS 6040 (Rev. 1/84)

Project Description:

Investigators:

B.J. Song	Senior Staff Fellow	LMMB, NIAAA
R.L. Veech	Chief	LMMB, NIAAA
T.L. Huh	Visiting Fellow	LMMB, NIAAA
Y.T. Chi	Visiting Fellow	LMMB, NIAAA

Objectives:

The specific aims of this project are to study the structure and regulation of the genes coding for pyruvate dehydrogenase (PDH) complex at a molecular level and to develop specific genetic probes for analysis of human specimens including human alcoholics.

Methods Employed:

Pyruvate dehydrogenase (PDH) complex is consisted of three catalytic enzymes (E_1: pyruvate dehydrogenase, E_2: lipoamide transacetylase, and E_3: lipoamide dehydrogenase) and two regulatory proteins (E_1 kinase and E_1 phosphatase). Based on the recently published sequences of PDH complexes, several oligodeoxynucleotides for respective subunit were synthesized and used to screen rat and human cDNA libraries by hybridization technique. Several clones were confirmed to be highly homologous to those reported earlier after nucleotide sequencing. The identified cDNA clones for these subunits of PDH gene complex were further utilized to identify full-length cDNA clones in rat and human cDNA libraries. The 5' end of the cDNA from each gene was prepared to screen the human genomic DNA library to study the gene structures. Peripheral lymphocytes or skin fibroblasts obtained from human individuals including alcoholics and grown in tissue culture will be analyzed for human genetic polymorphism using cloned PDH cDNA probe with appropriate immunochemical approaches.

Major findings:

1. We have cloned and confirmed our rat and human cDNA clones encoding for PDH E_{1x} and E_{1B} subunits by nucleotide sequencing.

2. We have also cloned and confirmed our rat and human cDNA clones for PDH E_3 subunit (lipoamide dehydrogenase).

3. However, the sequence of normal human PDH E_{1x} and E_{1B} clones have different sequences from those found in cancer cell lines such as HeLa or hepatoma cell lines.

4. Using full-length cDNAs for various PDH subunits, the regulation of these genes in human fibroblasts are being investigated.

Significance to Biomedical Research and the Program of the Institute:

Based on the recent results and proposals from our laboratory, the presence of 2,3-butanediol is closely associated with those who had alcohol in the past and that it is generated from conjugation of acetaldehyde which is a metabolic product of ethanol with hydroxyethylthiamine pyrophosphate on the pyruvate dehydrogenase multienzyme complex. However, 2,3-butanediol can still be detected in those people who had not drunk for quite sometime, indicating that the production of this compound may not totally be dependent on the level of circulating acetaldehyde but rather depend on the genetic makeups of each

individuals. The latter possibility will be carefully examined in an attempt to identify the molecular basis of individual differences in the level of 2,3-butanediol and to determine its potential as a genetic marker for human alcoholism as well as those who have problems of abnormal glucose metabolism such as patients of lactic acidosis with and without Leigh syndrome which is similar to Wernicke-Korsakoff encephalopathy in certain degree such as anatomical regions of affected brain areas. It is quite essential to the program of the Institute to study the research problems on human alcoholism by combination of molecular biology and immunochemical techniques.

Proposed Course:

Using full-length cDNAs for each PDH complex, subunit we are trying to express in bacteria system in order to study the characteristics of human enzymes which are very difficult to obtain and purify. By screening genomic DNA libraries, we will identify the transcription initiation sites and genomic organization of the PDH genes. The possibility of genetic polymorphism and differences in abnormal tissues will be also pursued by analyzing restriction fragment length polymorphism or specific ribonuclease digestion method for genomic DNAs in fibroblasts or lymphocytes obtained from human subjects including alcoholic and cancer patients.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00038-01 LMMB

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.)
Cerebral Blood Flow and Energy Metabolism in the Cat

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

P.I.:	A.C. McLaughlin	Section Chief	LMMB, NIAAA
Others:	E. Dora	Visiting Scientist	LMMB, NIAAA
	L. Ligeti	Visiting Scientist	LMMB, NIAAA
	T. Sinnwell	Technician	LMMB, NIAAA

COOPERATING UNITS (if any)

Department of Neurosurgery, Hospital of the University of Pennsylvania, Philadelphia (L. Sutton)

LAB/BRANCH
Laboratory of Metabolism and Molecular Biology

SECTION
Physical Chemistry

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.10	0.95	0.15

CHECK APPROPRIATE BOX(ES)

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

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

This study was undertaken to assess a new method for the non-invasive determination of regional cerebral blood flow without the use of radioactive tracers. Specifically, we investigated a new 19F NMR technique that has been used to measure the clearance of a fluorinated inert gas, CHF3 from the cerebral cortex of the cat. The 19F NMR technique was tested in three ways. First, the CO2- dependence of the measured cortical blood flows was investigated. Second, the cortical blood flow values calculated by 19F NMR were compared with cortical blood flow values determined simultaneously by radioactive microsphere techniques. Finally, the effects of CHF3 on cortical cerebral blood flow, and the short-term toxic effects of CHF3, were evaluated.

PHS 6040 (Rev. 1/84)

Project Description:

Investigators:

A.C. McLaughlin	Section Chief	LMMB, NIAAA
T. Sinnwell	Technician	LMMB, NIAAA
E. Dora	Visiting Scientist	LMMB, NIAAA
L. Ligeti	Visiting Scientist	LMMB, NIAAA
L. Sutton	Assistant Professor	Neurosurgery, Univ. of Penn.

Objectives:

The objective of this project is to develop a non-invasive technique for measuring regional cerebral blood flow in animals constrained inside the NMR magnet.

Methods Employed:

Our technique is based on the modified Kety-Schmidt approach, i.e., that blood flow is proportional to the rate constant for the washout of an inert diffusable tracer. We use ^{19}F NMR to measure the washout of a fluorinated inert gas, CHF_3, from the brain.

These studies also employed a more "classical" approach to measurement of blood flow, i.e., radioactive microspheres. Physiological parameters (e.g., PaO_2, $PaCO_2$, arterial pH, arterial blood pressure, etc.) were measured by standard techniques. Surgical procedures, e.g., cannulation of arteries, veins, intubation, etc., were standard procedures.

Major Findings:

The ^{19}F NMR technique was tested in two ways. First, the CO_2 dependence of the measured cortical blood flow values was investigated. Second, cortical blood flow values calculated by ^{19}F NMR were compared with blood flow values determined simultaneously by radioactive microsphere techniques.

Cerebral cortical blood flow values determined by ^{19}F NMR show the expected responsiveness to alterations in the pCO_2. Cerebral cortical blood flow values determined by ^{19}F NMR also correlated with cerebral cortical blood flow values determined by microsphere techniques, up to a value of 100 mls/100 gram/min. At higher flows, the values determined by NMR were substantially less than those determined by microspheres.

The present study demonstrates that ^{19}F NMR is a promising, non-invasive, technique for regional cerebral blood flow in experimental animals. However, it also points out a number of potential problems that must be investigated further before the technique can be used routinely. First, the the present work suggests that high concentrations of CHF_3 (70%) increase grey matter blood flow by approximately 35%, which is similar to the increase observed on inhalation of 35% xenon. The present study was performed with high concentrations of CHF_3 in order to optimize the signal-to-noise and minimize the sampling time (25 seconds). The future use of higher magnetic field strengths (i.e., 4.7 T) should increase the signal-to-noise, and permit the use of substantially lower CHF_3 concentrations. Presumably, the use of lower CHF_3 concentrations in the inhaled mixture will reduce the effect of CHF_3 on local cerebral blood flow rates, but this prediction remains to be demonstrated.

The present study also suggests that the apparent blood flow values calculated from CHF_3 washout data may be affected by diffusional limitations, even under conditions of mild hypercapnia. This possibility can be tested by repeating the experiments with another technique that is not affected by diffusional limitations (e.g., the original Kety-Schmidt approach).

The third potential problem is due to the washin of CHF_3 into non-cerebral tissue within the "sensitive volume" of the surface coil. For example, the present study suggests that the slow wash-out of CHF_3 from fat in the skull can cause an underestimate of the cortical blood flow rate by up to approximately 20%. This problem can be circumvented by using more refined spatial localization techniques for the collection of ^{19}F NMR signals. Any of the standard techniques that utilize magnetic field gradients can localize the ^{19}F NMR signal to a well-defined region of the brain, and avoid contamination from fat tissue in the skull, and other extracerebral tissue. This degree of spatial localization will also be necessary if the reflection of surface tissue from the skull is to be avoided, and the technique applied in a truly non-invasive manner to animals and, potentially, humans.

Significance to Biomedical Research and the Program of the Institute:

The ^{19}F NMR approach is potentially very useful for the study of regional cerebral blood flow. There are a number of advantages of this approach over existing techniques:

(1) It does not involve radioactive tracers.

(2) It is non-invasive, i.e., it can be used to perform sequential determinations of blood flow in an individual animal.

(3) It can be used in conjunction with in vivo NMR spectroscopy techniques.

(4) It has the potential for spatial localization of cerebral blood flow, using NMR imaging techniques.

The ^{19}F NMR approach is thus a useful technique, in conjunction with other in vivo NMR techniques, for studying the factors that control cerebral energy metabolism. It is also a potentially useful technique for studying regional cerebral blood flow in human patients. If the technique can be applied to humans, it could be a useful approach for determining the effects of chronic alcohol ingestion on regional cerebral blood flow and metabolism.

Proposed Course:

The ^{19}F NMR approach will be further tested. The "dose-response" curve of CHF_3 on the cerebral blood flow determined by ^{19}F NMR and the "classical" Kety-Schmidt approach will be determined. These experiments will establish the concentration of CHF_3 that does not significantly affect the cerebral blood flow, and also establish if diffusional limitations will complicate the interpretation of the results. We will also extend the ^{19}F NMR approach to another inert, diffusable, fluorinated gas, CH_3F. A radioactive form of this gas is currently being used for PET studies at NIH, and these studies should allow a direct comparison between PET and NMR approaches for regional blood flow determination.

Once the ^{19}F NMR technique is firmly established, it will be combined with other in vivo NMR techniques that give information on energy metabolism in the brain. These two techniques will then be used to study the factors that control the rate of energy metabolism in the brain.

Every effort will be made to extend the ^{19}F NMR technique to human subjects. This will involve the use of techniques that can spatially resolve ^{19}F NMR signals from different regions of the brain, and will also involve studies on the short and long-term effects of CHF_3 inhalation.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AA 0039-01 LMMB

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.)
Cerebral Blood Flow and Energy Metabolism in the Rat

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

P.I.:	A.C. McLaughlin	Section Chief	LMMB, NIAAA
Others:	M.T. Huang	Chemist	LMMB, NIAAA
	R. Werner	Veterinarian	NIAAA
	E. Dora	Visiting Scientist	LMMB, NIAAA
	L. Ligeti	Visiting Scientist	LMMB, NIAAA
	R. Lyon	Post Doctoral Fellow	LMMB, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Metabolism and Molecular Biology

SECTION
Physical Chemistry

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.65	1.65	

CHECK APPROPRIATE BOX(ES)
☐ (a) 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 developed a technique for measuring cortical cerebral blood flow in the rat. The technique is based on the original Kety-Schmidt approach, and involves cannulation of the jugular vein, the common carotid artery, the superior sagittal sinus, and, in some cases a femoral artery and vein. The technique has a number of advantages over other approaches. First, it does not require the calculation of the partition coefficient of the radioactive tracer used in the experiments. Second, it is not affected by possible diffusional limitations of the radioactive tracer. Third, it can be used to measure regional blood flow in the awake, unanesthetized animal.

Project Description:

Investigators:

A.C. McLaughlin	Section Chief	LMMB, NIAAA
M.T. Huang	Chemist	LMMB, NIAAA
R. Werner	Veterinarian	NIAAA
E. Dora	Visiting Fellow	LMMB, NIAAA
L. Ligeti	Visiting Scientist	LMMB, NIAAA
R. Lyon	Post Doctoral Fellow	LMMB, NIAAA

Objectives:

We are interested in determining regional cerebral blood flow in the rat, for the following two reasons:

(1) In conjunction with the arterial-venous difference for oxygen, the local cerebral blood flow can be used to calculate the local cerebral metabolic rate. We want to correlate the local cerebral metabolic rate with in vivo ^{31}P NMR studies of ATP, PCr, and Pi, and freeze clamped assays of other metabolites, to investigate the control of cerebral energy metabolism.

(2) We wanted to develop a regional cerebral blood flow technique that did not depend on unverifiable assumptions. This technique could then be used as the "gold standard" for testing a new ^{19}F NMR approach for regional cerebral blood flow.

To be useful for our purposes, the cerebral blood flow technique must have the following four properties:

(1) It can be used with rats,

(2) It can be used for regional blood flow determinations,

(3) It is not sensitive to the partition coefficient of the radioactive tracer employed, or to possible diffusional limitations of the tracer.

(4) It can measure regional cerebral blood flow on conscious, unanesthetized animals that are not under stress.

There are two approaches that satisfy the last three criteria - the original Kety-Schmidt approach and the "indicater-dilution" approach (e.g., radioactive microspheres). However, the radioactive microsphere technique does not satisfy the first criterion; i.e., it cannot be applied to small animals such as rats. We therefore chose the original Kety-Schmidt approach for these studies.

Methods Employed:

Catheters are inserted into the jugular vein, the common carotid artery and the sagittal sinus. Catheters are also inserted into the femoral artery and vein of anesthetized animals. Systemic blood pressure, arterial PaO_2, $PaCO_2$ and pH, and body temperature are monitored. Tritiated water is infused into the jugular vein at a constant rate. During this infusion, blood is withdrawn from the common carotid artery and the sagittal sinus vein at constant rates. At the end of three minutes, the animal is sacrificed with KCl, and the brain exposed. Either the whole cerebrum or the cortex are removed and counted for radioactivity, and the arterial and venous samples are counted for radioactivity. The ratio of the radioactivity in the brain to the arterial/venous difference in radioactivity gives directly the regional cerebral blood flow.

Major Findings:

Initial control experiments show that the technique does provide a valid measure of regional cerebral blood flow in the anesthetized and in the awake, unanesthetized rat. The physiological indicators confirm that, six hours after surgery, the rat has returned to an awake, non-stressed state. The observed blood flow values are reasonable, and show the appropriate responsiveness to variations in arterial CO_2 concentration. Preliminary experiments indicate that acute administration of ethanol (0.1%) causes approximately a 10% decrease in cortical cerebral blood flow.

Significance to Biomedical Research and the Program of the Institute:

In order to interpret many physiological experiments on the brain, the local cerebral metabolic rate must be known. In order to calculate the local cerebral metabolic rate, the regional cerebral blood flow must be known. The technique developed in this project provides this information.

Many physiological experiments require the use of anesthetics, which can affect cerebral blood flow and cerebral energy metabolism. The technique developed in this project provides a precise quantifications of these effects.

There are few blood flow techniques that can be performed on animals while they are in superconducting NMR magnets. The techniques that can be used in the magnet, e.g., the ^{19}F NMR approach, contain a number of assumptions that are difficult to verify. The blood flow techniques developed in this project provide an experimental test of these assumptions, and allow an objective evaluation of the ^{19}F NMR technique.

The classical Kety-Schmidt approach and the ^{19}F NMR approach are complementary. For example, the ^{19}F NMR approach works well with large animals, such as cats, but is difficult to use with small animals such as rats. In contrast, the classical Kety-Schmidt approach works well with rats or cats. The ^{19}F NMR approach is non-invasive (in principle), and gives a continuous read-out of blood flow in an individual animal. In contrast, the classical Kety-Schmidt approach is invasive, and can only give one experimental point per animal.

The technique developed in this project will be useful for studying the effects of alcohol on regional cerebral blood flow and energy metabolism. In this respect, it is important that the technique can be used in the unanesthetized animal, so that the effects of alcohol are not obscured by either direct effects of the anesthetic, or a synergistic interaction between alcohol and the anesthetic. In its present form, the technique gives directly the cerebral cortical blood flow. However, it can be modified to provide information on blood flow to many other localized regions of the brain.

Proposed Course:

The technique developed in this project will be used extensively. First, it will be used in conjunction with ^{31}P NMR experiments and freeze-clamped metabolite assays in rats, to study the control of cerebral energy metabolism. Second, it will be used to test the ^{19}F NMR technique for determining regional cerebral blood flow. Third, it will be used to study the effect of acute and chronic alcohol ingestion on regional cerebral blood flow and metabolic rate.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA 00040-01 LMMB

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.)
Electrostatic Properties of Membranes

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

P.I.:	A.C. McLaughlin	Section Chief	LMMB, NIAAA
Other:	K. Hines	Technician	LMMB, NIAAA

COOPERATING UNITS (if any)
Physiology Department, State University of New York, Stonybrook, New York (S. McLaughlin); Biochemistry Department, University of Pennsylvania, Philadelphia, PA (J.R. Williamson).

LAB/BRANCH
Laboratory of Metabolism and Molecular Biology

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.85	0.75	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 surface potential of cellular membranes is an important determinant in the physiological function of the cell. We have investigated a number of factors that affect the surface potential of membranes. We have also modified the theory that has been used to calculate the surface potential, the Gouy-Chapman theory, to account for these factors.

Triphosphoinositide lipids in plasma membranes could have up to five negative charges. We have investigated the interaction of calcium, magnesium, potassium, protons and other cations with triphosphoinositides, and determined the number of cations bound to the lipid under physiologically-relevent conditions.

PHS 6040 (Rev. 1/84)

Project Description:

Investigators:

A.C. McLaughlin	Section Chief	LMMB, NIAAA
K. Hines	Technician	LMMB, NIAAA
S. McLaughlin	Professor	Physiology, SUNY, Stonybrook, NY
J.R. Williamson	Professor	Biochemistry, University of PA

Objectives:

This project has two objectives. The first objective is to investigate the electrostatic potential near a charged membrane, and to determine how the structure of the charged groups at the surface affect the potential. The second objective is to quantify the binding of calcium to charged components present at the surface of biological membranes, i.e., sialoglycolipids and triphosphoinositide lipids.

Methods Employed:

We used classical surface chemistry techniques to determine the zeta potential, fluorescent techniques to determine the electrostatic potential 10 A from the surface of the membrane, and NMR techniques to determine the electrostatic potential at the surface of the membrane. We also used NMR techniques to investigate the protonation of phosphomonoester groups in polyphosphoinositides.

Major Findings:

The Gouy-Chapman theory predicts that if charged groups extend from the surface of the membrane, the magnitude of the electrostatic potential at the surface will be reduced, and the spatial profile of the potential will be altered. We have confirmed this prediction with sialoglycolipids (gangliosides), in which the charged sialic acid residues extend approximately 10 A from the surface.

Our studies indicate that, under physiologically-relevant conditions, calcium binds very weakly to gangliosides on the outer surface of cellular plasma membranes, and very weakly to triphosphoinositide lipids on the inner surface of cellular plasma membranes. However, the studies suggest that under physiological conditions, potassium binds strongly to triphosphoinositides, and that there is a cooperative interaction between the two phosphomonoester groups in triphosphoinositides.

Significance to Biomedical Research and the Programs of the Institute:

A number of important physiological functions of cellular membranes are dependent on the surface potential at the membrane-solution interface. This is particulary true for membrane functions that depend directly on the transmembrane potential difference, because the transmembrane potential is sensitive to changes in the surface potential. For example, it is now accepted that the effect of calcium and other divalent cations in shifting the current/voltage relationship of nerve is due to the effect of calcium on the surface potential of the nerve membrane. However, the interpretation of this, and many other, effects has been complicated by the fact that the theory that has been used to calculate the surface potential (the Gouy-Chapman theory) is obviously oversimplified, and may not be relevant to biological membranes. In the last five years we have proposed a number of modifications to the Gouy-Chapman theory and tested them on membranes reconstituted with components isolated from biological membranes. Part of

the present project concerns one of the modifications to the theory - e.g., the effect of the detailed structure of the charged groups on the surface potential, and the effect of monovalent cation binding on the surface potential. Our results help to explain two apparent paradoxes. First, the surface potential of cells is known to be substantially smaller than the value calculated from the simple Gouy-Chapman theory, assuming the known density of charged sialic acid residues. Second, the surface potential of model phospholipid membranes containing titratable residues, i.e., the phosphomonoester groups in polyphosphoinositides, does not change significantly as the groups are titrated.

The observation of cooperative binding of protons to the phosphomonester groups of polyphosphoinositides probably has no physiological significance, but it is important in understanding the physical chemistry of the inositide lipids, and in assigning the ^{31}P NMR spectra of these intermediates. The usual approach to assigning the ^{31}P NMR signals is to follow the shifts in the signals as a function of pH. However, for a number of the polyphosphoinositide intermediates, the pH dependence of the ^{31}P NMR spectra are bizarre, and the assignment of the signals difficult. For example, for tetraphosphoinositol compounds which contain at least three adjacent phosphomonester groups (i.e., 3,4,5, tetraphosphoinositide) some of the phosphomonoester groups actually become deprononated as the pH is lowered. We were, however, able to interpret quantitatively the pH titrations of these compounds in terms of cooperative interactions between the phosphomonoester groups. The analysis of the data in this manner made the assignment of the NMR spectra straightforward.

Proposed Course:

We will extend our experiments with sialoglycolipids to sialoglycoproteins. We have developed a purification procedure for glycophorin, the major sialoglycoprotein in the erythrocyte membrane, and a procedure for the assymmetric incorporation of the protein into well-defined phospholipid vesicles. We will also extend our studies on the competition between potassium and other ions for the phosphomonoester binding sites in triphosphoinositides by using ^{39}K NMR. Finally, we plan to study the interaction of triphosphoinositides with glycophorin. Initial reports indicate that triphosphoinositides interact strongly with glycophorin. This system might then be a good model system for studying the interaction of inositol lipids with membrane proteins.

Publications:

Toner M, Vaio G, McLaughlin A, McLaughlin S. The adsorption of cations to phosphatidylinositol 4,5-bisphosphate, Biochemistry. in press.

Langer M, Winiski A, Eisenberg M, McLaughlin A, McLaughlin S. The electrostatic potential adjacent to bilayer membranes containing either charged phospholipids or gangliosides. In: Ledeen R, Tettamanti G, Yu R, Hogan E, Yates A, eds. New Trends in Ganglioside Research: Neurochemical and Neuroregenerative Aspects. Springer Verlag: New York. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00041-01 LMMB

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 Plasma Free Magnesium Concentration by Ion-Selective Electrodes.

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

P.I.: A.C. McLaughlin Section Chief LMMB, NIAAA

Other: K. Hines Technician LMMB, NIAAA

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Metabolism and Molecular Biology

SECTION

Physical Chemistry

INSTITUTE AND LOCATION

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0.75	0.75	

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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.)

An ion-selective technique has been developed for the determination of free serum magnesium levels. A number of major technical difficulties have been overcome, but further studies to determine the accuracy of the technique are necessary.

PHS 6040 (Rev. 1/84)

Project Description:

Investigators:

A.C. McLaughlin	Section Chief	LMMB, NIAAA
K. Hines	Technician	LMMB, NIAAA

Objectives:

A number of approaches have been utilized to determine the plasma free magnesium concentration. All of these approaches are indirect. For example, one approach relies on the determination of the plasma total magnesium concentration, and an estimation of the fraction of magnesium bound to serum proteins and small anions. Another approach relies on the extrusion of plasma through a semipermeable membrane. All of the techniques are subject to potentially large errors. The purpose of this study is to develop and test an ion-selective electrode approach for measuring directly the plasma free magnesium concentration.

Methods Employed:

The ideal approach for determining free magnesium concentrations would utilize a specific magnesium-sensitive electrode that was insensitive to other ions. Unfortunately, this electrode does not exist. However, there are commercially-available electrodes that are sensitive to both magnesium and calcium. Our protocol was to use two electrodes - a non-specific divalent cation electrode that was sensitive to both magnesium and calcium, and a specific calcium electrode that was not sensitive to magnesium. By comparing the results from both the electrodes, the free magnesium concentration can, in principle, be determined. We also used a commercial blood-gas analyzer to determine the free bicarbonate, sodium and potassium concentrations.

Major Findings:

The major technical problems encountered were:

(1) The non-specific divalent cation electrode has substantial drift in the voltage output, probably because of substantial anion permeability of the semipermeable membrane. This problem was overcome by measuring a reference solution before and after every sample determination, and calculating directly the drift in the voltage output.

(2) The voltage output of the non-specific divalent cation electrode was sensitive not only to magnesium and calcium, but also to sodium, potassium and bicarbonate concentrations. This problem was overcome, to some extent, by determining the free concentration of all of these components and obtaining the magnesium titration curve under these conditions. A potential problem with this approach is that relatively small variations in the free concentrations of calcium, sodium and bicarbonate can cause produce substantial shifts in the magnesium calibration curve. The accuracy of the final calculated value of the free magnesium concentration therefore, depends very sensitively on the accuracy of the determination of the concentration of all these other components.

Preliminary results give a free magnesium concentration of 0.47 mM, and a free calcium concentration of 1.1 mM in one sample of human plasma. These values are reasonable, but we do not yet have enough data to estimate the error in the calculation.

Significance to Biomedical Research and the Program of the Institute:

The serum magnesium level is an important controlling factor in many physiological functions. Many pathological situations, i.e., withdrawal from chronic alcohol intoxication, appear to result in hypomagnesemia, but it is not clear if the plasma free magnesium level is altered. If the new approach we have developed does prove to be accurate, it can be used to determine the plasma free magnesium levels in patients under these conditions.

Proposed Course:

Work will continue on an analysis of errors in the technique to determine the overall accuracy of the calculated free magnesium level. If the technique proves feasible, we will use it to determine plasma free magnesium levels in hypomagnesemic patients.

Publications:

None.

Annual Report of the
Laboratory for Physiologic and Pharmacologic Studies
Division of Intramural Clinical and Biological Research
National Institute on Alcohol Abuse and Alcoholism
October 1, 1987 - September 30, 1988
George Kunos, M.D., Ph.D., Chief

Introduction

The Laboratory of Physiologic and Pharmacologic Studies (LPPS) was established in fiscal year 1986, and new Chiefs of the Laboratory and of the Immunology Section were recruited in the Fall of 1987. Research in the Laboratory as a whole is focussed on the cellular mechanisms involved in neurohormonal signal transduction and on the role of these processes in the biological effects of ethanol. Scientists in the Laboratory are using biochemical, neurophysiological, electrophysiological and molecular biological approaches to address questions within this general theme. The Laboratory consists of four research groups: three Sections and the Office of the Chief. During fiscal year 1988, the Sections on Receptor Mechanisms and Electrophysiology have made further progress in their well-established research areas as discussed below. Current work in the Section on Immunology focuses on the physiology and molecular biology of phosphoprotein metabolism and calcium-regulated signaling pathways in neural and lymphoid tissue, with the aim of extending these studies to examining the role of these pathways in the biological effects of ethanol and in the pathomechanisms of AIDS. Finally, work in the newly created Office of the Chief is aimed at clarifying mechanisms of heterologous regulation of adrenergic receptors in liver and lung tissue, as potential targets for the effects of ethanol on the cell membrane. Other studies examine the role of brainstem neuromechanisms in the control of blood pressure and in ethanol-induced hypertension.

I. Office of the Chief

The Office of the Chief has been newly established to study neurotransmitter receptor mechanisms which are potential targets for the biological effects of ethanol. Within this broad theme, research is focused on three specific areas: mechanism of the inverse regulation of cyclic AMP-linked beta- and calcium-linked alpha-adrenergic receptors; the role of the immune system in the regulation of pulmonary beta-adrenergic receptors; and the role of neuronal systems of the brainstem in the regulation of blood pressure and in ethanol-induced hypertension.

a. Mechanisms of the inverse regulation of hepatic alpha- and beta-adrenergic receptors:

Hormones and neurotransmitters can usually interact with more than one type of receptor, coupled to different

transduction pathways, to produce their biological effects. Studies in the rat liver have demonstrated that selection of the functionally dominant adrenergic receptor type (cyclic AMP-linked beta- or calcium-linked alpha-receptor) is linked to the process of cell differentiation. There is also evidence to suggest that the inverse, reciprocal changes in alpha and beta-adrenergic responses observed in a variety of conditions is due to corresponding changes in the coupling of these receptors to their post-receptor pathways. These changes appear to involve at least two membrane enzyme systems: phospholipase A2 and protein kinase C, suggesting complex interactions among various membrane proteins. Recent evidence indicates that ethanol can interfere, in a highly selective manner, with receptor/receptor interactions in the cell membrane. Ongoing studies will therefore further clarify the mechanisms of the coupled regulation of hepatic alpha and beta-adrenergic receptors, and examine whether the interaction between these two membrane receptor systems may be a target for the action of ethanol.

b. The role of brainstem neuromechanisms in blood pressure regulation and in the cardiovascular effects of ethanol:

The hypertensive action of chronic ethanol consumption has been well established in population and clinical studies. There is also evidence to suggest that ethanol-induced hypertension in a rat model is related to impaired baroreflex activity. Our previous studies have demonstrated an interaction between alpha 2-adrenergic and endorphinergic neuronal systems in the brainstem both of which can facilitate the depressor baroreflex response. Preliminary results indicate the existence of a GABA-ergic pressor mechanism, which appears to be linked to the above two neuronal systems. Since all of the above neurotransmitter systems in the brain are known to be influenced by ethanol, we will examine whether they are involved in the cardiovascular effects of ethanol in rats.

c. A novel interaction between the immune system and the autonomic nervous system:

Heterologous regulation of adrenergic receptors has important implications for the pathomechanism and therapy of certain disease conditions. For example, the therapeutic effectiveness of glucocorticoids in bronchial asthma has been attributed to their ability to upregulate beta-adrenergic receptors in the lung. We have recently discovered that interleukin-1, as well as some unidentified lymphokines, have a similar effect, which is synergistic with the effects of glucocorticoids. Further studies will be aimed at identifying the additional lymphokines involved in this effect, establishing the mechanism of their synergistic interaction with glucocorticoids, and testing whether the known effects of ethanol on immune function also

involve an interference with this novel lymphokine/beta-adrenergic interaction.

II. Immunology Section

The Section on Immunology was recently reorganized to examine immunological and neurobiological consequences of alcohol abuse and alcoholism (i.e., intoxication, dependence and withdrawal) and of disorders of the immune system, such as AIDS. Since these pathologies may reflect the impairment or alteration of normal signal transduction events, investigations are being carried out on the fundamental mechanisms by which lymphoid and neural cells respond to effectors. Research focuses on the role of cyclic nucleotide and phosphoprotein metabolism in such "signaling pathways" and the regulation of these by Ca^{2+} and calmodulin. The Section emphasizes the use of molecular biological approaches, in concert with classical biochemical and immunological methods, to study the regulation of these systems. Presently, the Section is recruiting candidates from a variety of disciplines in order to assemble an integrated research team. The following summary provides a brief overview of the plans for our future work and is divided into three main areas:

a. Molecular biology of calmodulin-dependent signal transduction pathways:

Intracellular signaling events are central to modulating the biochemical response of excitable cells. Calmodulin and several enzymes that it regulates (i.e., cyclic nucleotide phosphodiesterase, phosphoprotein phosphatase) have their highest concentrations in brain, suggesting an important role in neuronal communication. Previous studies have shown that expression of such enzymes is very selective and linked to neuronal development. Similarly, differential expression of calmodulin-dependent phosphatase in cells of lymphoid origin indicates that Ca^{2+}-regulated signaling events occurring during cell activation may require particular phosphatases. In order to understand the molecular basis of such regulation, we have cloned cDNAs for the phosphatase and are currently characterizing putative clones for the phosphodiesterase. These studies will greatly extend our understanding of signal transduction events.

b. Modulation of phosphoprotein metabolism during signaling events:

Control of protein phosphorylation is a well-established mechanism for regulation of intermediary metabolism, cytoskeletal organization and receptor function. We have initiated studies to prepare and utilize specific immunologic probes against important phosphorylated sites. In so doing, the status of phosphorylation of a particular component (e.g., a growth factor receptor or cytoskeletal component) can be directly assessed after challenge of the

cell or organism with biological effectors. Furthermore, the use of immobilized antibodies to certain "consensus" phosphorylation sites may enable the detection and characterization of novel proteins, the expression of which can be correlated with specific experimental conditions or pathologies. Thus, in addition to providing a means for analysis of signaling events, this approach may also be of diagnostic or predictive value.

c. Involvement of signaling events in alcoholism and pathologies affecting the nervous and immune systems:

The participation of signal transduction in the etiology of diseases such as alcoholism and AIDS has not been examined in detail. However, it seems likely that disturbances in the "processing" of information are involved, causally or as reflected by symptoms, in the underlying basis of these diseases. Since we intend to make a broad effort to characterize many of the components involved in signaling in neural and lymphoid tissue, we are well-positioned to detect alterations in component function that can be correlated with a specific pathology. Using murine models for alcoholism, we plan to compare the biochemical elements of signal transduction pathways in normal and experimental tissue. Similarly, using inbred strains of mice that exhibit neuropathologies, we will use immunocytochemical and molecular biological methods to assess whether defects in the signaling pathways may be present. Finally, by challenging isolated murine T-cells with purified surface components from HIV particles, we can examine cyclic nucleotide and phosphoprotein metabolism under normal and immunomodulated conditions. The information obtained may prove useful in assessing the involvement of signaling events in the pathology of other disease states.

III. Section of Electrophysiology

The research program of the Section of Electrophysiology is directed toward elucidating the cellular mechanisms involved in the acute and chronic actions of ethanol in the nervous system. Although the effects of ethanol on behavior are well known, the cellular mechanisms by which ethanol produces its effects on nervous system function are poorly understood. The investigations of the Section use modern electrophysiological, biophysical and optical methods to study the cellular and molecular mechanisms involved in ethanol's actions in nervous tissue. The research program of the Section encompasses investigations in two broad areas: nerve cell excitability and neurosecretory mechanisms. These two areas provide the organizational basis of this report.

a. Nerve cell excitability and ion channels:

Previous investigations have demonstrated that ethanol can

alter the excitability of neurons in the central nervous system; however, the mechanisms involved in these effects are not known. In order to determine the alterations of ion channel function that underlie the effect of ethanol on nerve cell excitability, we have developed new experimental preparations (acutely isolated adult mammalian neurons) and applied recently developed biophysical methods (patch-clamp and single-electrode voltage-clamp recording) to investigate membrane ion channel function in mammalian neurons. We have identified and are characterizing the following ion currents in mammalian neurons: two sodium currents (tetrodotoxin-sensitive and tetrodotoxin-resistant) two calcium currents (transient and sustained), a calcium-activated chloride current and three potassium currents (transient, delayed rectifier and calcium-activated). These ion currents underlie action potential generation and steady-state membrane excitability in mammalian neurons. In addition, we are also studying receptor gated ion channels. In order to determine how ethanol affects nerve cell excitability, we are investigating the effects of ethanol on these ions currents. These studies should increase our understanding of the mechanisms involved in ethanol's actions on nervous system excitability.

b. Neurotransmitter release and neurosecretion:

Previous studies have shown that ethanol can alter neurotransmitter release and neurosecretion, but the mechanisms involved in these effects have not been determined. It is well established that calcium ions play an essential role in the neurosecretory process. To investigate whether the effects of ethanol on neurosecretion result from alterations in cellular calcium metabolism, we are applying recently developed biophysical and optical methods. Using the patch-clamp recording technique, we are studying the cellular mechanisms that modulate membrane calcium current that triggers neurosecretion, and the effects of ethanol on those mechanisms. The studies indicate that the calcium channel may be regulated by protein kinase C. We are also studying the role of intracellular calcium ions in the neurosecretory process using optical methods with the fluorescent calcium-indicator, Quin 2, and have found a receptor mediated elevation of inositol triphosphate and intracellular calcium levels and a release of neurotransmitter. Analysis of these phenomena indicates that the neurotransmitter release is associated with an inositol triphosphate-induced mobilization of intracellular calcium ions. Ethanol inhibits this receptor mediated release of neurotransmitter. Current experiments are directed toward elucidating the site of ethanol's action in this neurosecretory process by examining the effects of ethanol on the interrelationship between intracellular

calcium mobilization, phosphoinositide metabolism and neurotransmitter release. Characterization of neurosecretory mechanisms and the actions of ethanol on those processes should increase our understanding of the mechanisms involved in ethanol's actions on neurotransmitter release and neurosecretion.

IV. Section on Receptor Mechanisms

The overall goal of the studies being performed within the Section on Receptor Mechanisms continues to be a determination of the neurochemical mechanisms responsible for neuroadaptive responses to ethanol (functional tolerance and physical dependence). Our research supports the postulate that neurotransmitter and neuromodulator receptors, and particularly receptor-effector coupling processes, are sensitive to disruption by ethanol. Our investigations apply biochemical and molecular biological techniques to identify the initial response to ethanol of these systems as well as adaptations and/or pathological changes that occur following chronic ethanol exposure or ingestion. These changes, in addition to elucidating the mechanism of action of ethanol in the CNS, may prove useful as potential indicators of chronic alcohol consumption.

Another approach to elucidating the mechanisms underlying neuroadaptation to ethanol is to focus on neuronal systems and neuromodulators that influence various aspects of ethanol tolerance. We have previously postulated the existence of intrinsic and extrinsic neuronal systems that affect tolerance. Extrinsic systems are those which modulate the acquisition, expression or dissipation of tolerance, while intrinsic systems are those in which tolerance to specific effects of ethanol is actually encoded, presumably by changes in synaptic efficacy. This framework has also been used to describe the neurobiology of learning and memory processes which, like tolerance, represent adaptation of the CNS to external stimuli. The major focus of our recent work is the neurohypophyseal hormone, arginine vasopressin (AVP). This neuropeptide appears to represent an extrinsic system that modulates ethanol tolerance, and behavioral, biochemical and molecular biological techniques are being used to investigate the mechanism and sites of action of AVP in the CNS, as well as the effects of ethanol on regulation of AVP biosynthesis and release. Definition of the role of a naturally-occurring hormone in ethanol tolerance may lead to development of therapies to manipulate tolerance, and, as a consequence, alcohol drinking behavior.

a. Receptor-effector coupling processes:

In brain, ethanol has a selective, region-specific effect on the function of G_s, the stimulatory guanine nucleotide binding protein. After chronic ethanol ingestion that results in ethanol tolerance and physical dependence, the amount of cerebral cortical G_s appears to be decreased,

based on changes in cholera toxin-induced ribosylation and on initial Northern blot analyses. This change in G_s may underlie our previous findings of decreased responsiveness of cerebral cortical adenylate cyclase (AC) to stimulation by various agonists, and of changes in beta-adrenergic agonist binding to cerebral cortical membranes in ethanol-fed mice. The alteration in cerebral cortical G_s is selective, in that no significant changes in pertussis toxin-catalyzed ADP-ribosylation, or in synthesis of G_{oalpha} measured on Northern blots, were observed in ethanol-fed mice. Although a decrease in G_{ialpha} mRNA was observed, this change did not appear to be reflected functionally in a change in adenylate cyclase inhibition. In the hippocampus of ethanol-fed mice, changes were found in adenylate cyclase (AC) activity and in beta-adrenergic agonist binding properties that were similar to those in cerebral cortex, suggesting that the amount or function of hippocampal G_s may also be altered by chronic ethanol ingestion. However, in cerebellum, few changes were observed. This regional specificity in the effects of ethanol on receptor-AC coupling was supported by autoradiographic studies of high-affinity forskolin binding in brains of control and ethanol-fed mice. The selective and region-specific alterations in CNS signal transduction mechanisms produced by chronic ethanol ingestion are expected to result in significant disruption of neuronal function, which may be associated with specific behavioral and physiological consequences. The changes in receptor-effector coupling systems may be related to ethanol tolerance and/or physical dependence, or may represent pathological effects of ethanol.

The ethanol-induced changes observed in receptor-AC coupling in brain suggested that this system might represent an objective biochemical marker of ethanol consumption. Studies of platelets of control and alcoholic subjects revealed decreased stimulation of AC activity by guanine nucleotides and other agonists in the platelet membranes of the alcoholics. In addition, monoamine oxidase (MAO) activity, and its <u>in vitro</u> inhibition by ethanol, were examined in platelets of alcoholics and controls. MAO activity in platelets of alcoholics was significantly more susceptible to inhibition by ethanol. Statistical analysis demonstrated that the differences in platelet enzyme characteristics were not associated with age, race, smoking or illicit drug use, and that there was no significant correlation with the duration of problems with alcohol. Differences in platelet enzyme activities were long-lasting; fluoride-stimulated AC activity was lower in alcoholic subjects who had abstained from alcohol for one to four years. Discriminant analysis showed that the use of values for the inhibition of MAO activity by ethanol and fluoride-stimulated AC activity correctly classified 75% of alcoholics and 73% of controls. These measures are,

therefore, reasonably sensitive and specific and may be helpful, in combination with other biochemical variables, in identifying subjects who can be classified as alcoholics or, possibly, those with a predisposition to alcoholism.

The acute effects of ethanol on receptor-coupled AC activity were studied in cultured cells. While these systems are not influenced by physiological interactions that occur in vivo, and cannot be used to measure behavioral aspects of adaptation, they have the advantages of relative homogeneity, and the possibility for directly elucidating biochemical mechanisms of action of ethanol. In PC12 cells, it was found that ethanol increased agonist-stimulated AC activity in cell membranes, similar to its effect on brain cell membranes. However, in the intact cells, ethanol decreased cyclic AMP levels, and inhibited AC activity (measured by conversion of ^{3}H-ATP to ^{3}H-cyclic AMP). The inhibitory effects of ethanol were not mediated by G_i or by protein kinase C. In a second subclone of PC12 cells, ethanol also increased AC activity in cell membranes, but increased agonist-stimulated cyclic AMP levels in the intact cells. Thus, it appears that, while ethanol can clearly stimulate membrane-bound AC activity, the acute effect of ethanol on cyclic AMP levels and AC activity in intact cells varies, depending on the regulatory mechanisms that influence AC activity in the whole cell. These data indicate that extrapolation between various cell culture systems, and from in vitro to in vivo systems, must be cautious.

Primary cell cultures from brain may be more similar to brain cells than are tumor cells or transformed cell lines. A primary culture of cerebellar granule cells has been used to examine the effect of ethanol on glutamate receptor-effector coupling processes. Low concentrations of ethanol inhibited cyclic GMP production stimulated by glutamate acting at the kainate and NMDA receptor, while ethanol had less effect on the stimulation of cyclic GMP production by atrial natriuretic factor, which activates particulate guanylate cyclase. Glutamate, on the other hand, stimulates soluble guanylate cyclase in a calcium-dependent manner, and preliminary studies suggest that glutamate-stimulated calcium flux in cerebellar granule cells is very sensitive to inhibition by ethanol. Inhibition by ethanol of biochemical responses to glutamate suggests a novel mechanism for the depressant effects of ethanol, and alterations in NMDA receptor-effector coupling processes may also be involved in adaptation to ethanol, since activity of NMDA-coupled calcium channels has been implicated in long-term neuroadaptation.

b. Extrinsic systems that modulate ethanol tolerance: vasopressin:

By using agonists and antagonists that are selective for V_1 and V_2 vasopressin receptor subtypes, the brain receptors mediating the ability of vasopressin to maintain ethanol tolerance have been defined as V_1. Autoradiographic studies have identified vasopressin binding sites with characteristics of V_1 receptors in lateral septum, hippocampus and several other brain areas. A portion of the receptors in the lateral septum may be localized presynaptically on terminals of noradrenergic neurons, since the number of receptors was reduced after 6-OHDA treatment. We previously showed that intact noradrenergic systems are required in order for vasopressin to maintain ethanol tolerance, and these results suggest that vasopressin may act at presynaptic V_1 receptors to modulate neurotransmitter release, and thus affect tolerance. Another possible (postsynaptic) mechanism of action is suggested by studies of the protooncogene, c-fos. This gene is involved in growth and differentiation, and has been postulated to play a role in learning and memory (i.e., long-term adaptation). Its expression can be stimulated by neurotransmitters and by increases in intracellular calcium. Vasopressin, acting at peripheral V_1 receptors, increases intracellular calcium levels, and may produce this effect in the CNS. We have found that vasopressin, administered intracerebroventricularly, increases c-fos expression in the lateral septum and hippocampus, as measured on Northern blots. V_1 agonists are also effective, while V_2 agonists are less active. The increase in c-fos expression may be a direct or indirect (e.g., through neurotransmitter release) effect of vasopressin, and suggests a mechanism by which the peptide may modulate tolerance as well as other neuroadaptive processes such as learning and memory.

Behavioral studies with vasopressin receptor antagonists suggested that the endogenous hormone, interacting with CNS V_1 receptors, plays a role in the maintenance of ethanol tolerance. These findings led us to investigate the effect of chronic ethanol exposure on vasopressin synthesis and release. In rats exposed chronically to ethanol by vapor inhalation, as well as in mice chronically fed ethanol in a liquid diet, hypothalamic vasopressin mRNA levels were decreased. The results were obtained using Northern blots, as well as <u>in situ</u> hybridization, by which dramatic decreases were observed. The effect of chronic ethanol ingestion on vasopressin levels in plasma varied, depending on the method used for ethanol exposure. The data suggested that stress and/or dehydration can interfere with measurements of the effects of ethanol on vasopressin release and levels. However, it seems clear that ethanol decreases vasopressin synthesis, and interferes with the normal feedback control of this synthesis. The decrease in vasopressin synthesis after chronic ethanol ingestion may have important physiological consequences in terms of an individual's ability to respond to various stimuli.

Although a decrease in vasopressin synthesis may seem paradoxical with regard to the role of the peptide in maintaining tolerance, it is not necessary for peptide synthesis to be increased in order for it to modulate tolerance. Furthermore, a number of vasopressin-containing neuronal pathways have been described, and vasopressin in brain, as opposed to the hypothalamic hormone, may be most important for the modulation of tolerance. The effect of chronic ethanol ingestion on synthesis of vasopressin in various brain areas is under investigation using in situ hybridization techniques.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00401-01 LPPS

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.)

Interaction between the immune system and adrenergic receptors

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

P.I.:	G. Kunos	Chief	LPPS, NIAAA
Others:	M. Virmani	Research Chemist	LPPS, NIAAA
	T. Nakane	Visiting Fellow	LPPS, NIAAA

COOPERATING UNITS (if any)

J. Oppenheim, LMI, FCRF, NCI, Frederick, MD

LAB/BRANCH
Laboratory of Physiologic and Pharmacologic Studies

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TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.9	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.)

An area of major interest to us is the mechanisms involved in heterologous regulation of adrenergic receptors and the relevance of such regulation to disease conditions. Loss of beta-adrenergic receptors and receptor reactivity is a pathogenic feature in bronchial asthma. Glucocorticoids are effective in the treatment of asthma, which has been attributed to their ability to upregulate pulmonary beta-adrenergic receptors. In a study of glucocorticoid-beta-adrenergic interactions in cultured human lung cells we made the accidental observation that the beta-receptors of these cells were upregulated by serum-free medium conditioned with EBV-transformed human lymphocytes. The effect of the conditioned medium (LCM) could be attributed to a mixture of proteins, with approximate molecular weights of 70, 35 and 15 kD. Glucocorticoids also increased the density of beta-receptors in lung cells, and markedly potentiated the similar action of LCM. Human monocytic interleukin-1 (IL-1) as well as recombinant IL-1alpha and IL-1beta also upregulated beta-receptors in the lung cells, and this effect was similarly potentiated by glucocorticoids. A neutralizing antibody against IL-1 blocked the effect of the 15 kD protein from LCM, but not the effects of the 35 and 70 kD proteins. These results indicate that lymphocytes release protein factors including IL-1, which up-regulate pulmonary beta-adrenergic receptors. As the major source of IL-1 in the body is macrophages including pulmonary macrophages, these findings suggest the existence of a novel, paracrine mechanism for the regulation of beta-receptors in the lung.

Project Description

Investigators:

G. Kunos	Chief	LPPS, NIAAA
W. Laskey	Visiting Scientist	LPPS, NIAAA
J. Mastrianni	Staff Fellow	LPPS, NIAAA
S. Zakhari	Executive Secretary	NIAAA

Objectives:

The initial objective is to develop a reproducible rat model for ethanol-induced hypertension based on information in the literature. This model will then be used to test whether chronic ethanol consumption can cause changes in the cardiovascular effects of β-endorphin, α2-adrenergic agonists and GABAergic agents at the level of the brainstem and spinal cord. The use of the appropriate selective antagonists or selective antisera can help to unmask any effect of ethanol on tonically active systems.

Methods:

Normotensive Sprague-Dawley and Wistar rats will be chronically treated with ethanol for 8-12 weeks, according to the protocol of Chan & Sutter (Canad. J. Physiol. Pharmac. 60:777, 1986). Induction of anesthesia, direct measurements of blood pressure and heart rate, and central administration of drugs will be done as described earlier (Mosqueda and Kunos, PNAS 84:8637, 1987). Baroreflex sensitivity will be assessed from the bradycardic response to systemic administration of a vasopressor or to electrical stimulation of the aortic nerve.

Major Findings:

In an earler study at McGill University, published during FY-88 (publication #1), we examined which opiate receptor subtype is involved in the hypotensive and bradycardic effects of clonidine and β-endorphin in urethane-anesthetized rats. Both the agonists and the various antagonists were microinjected directly into the nucleus tractus solitarii (NTS). In spontaneously hypertensive rats (SHR) and in Doca-salt hypertensive Sprague-Dawley rats the hypotensive and bradycardic effects of both clonidine (5 nmol) and β-endorphin (280 fmol) were inhibited by naloxone (270 pmol) or the δ-receptor antagonist, ICI-174864 (270 pmol), but not by the μ-receptor antagonist, β-funaltrexamine (270 pmol). In normotensive Sprague-Dawley rats the effect of clonidine and β-endorphin were similar, but they were inhibited by naloxone or β-funaltrexamine and not by the δ-antagonist, ICI-174864. These results support the hypothesis that β-endorphin release and subsequent stimulation of opiate receptors in the NTS are involved in the cardiovascular effects of clonidine in rats (Mosqueda et al., Endocrinology 118:1814, 1986). They further suggest, however, that hypertension induces a change in the subtype of opiate receptor that mediates these effects.

In another study (publication #2) we addressed the question whether or not peripheral opiate receptors also contribute to the effects of clonidine. In urethane-anesthetized, normotensive Sprague-Dawley rats clonidine given intravenously (5 μg/kg) or intra-NTS (5 nmol) caused hypotension and bradycardia, which were dose-dependently inhibited by intra-NTS administration of either naloxone (ID50: 29 ng) or its water soluble analog, naloxone

methylbromide (ID50: 316 ng). However, when the antagonists were given i.v. only naloxone (2 mg/kg) produced inhibition, while naloxone methylbromide was ineffective even at the dose of 20 mg/kg, given to offset its lower potency at opiate receptors. We concluded that only central and not peripheral opiate receptors are involved in the cardiovascular depressor response to clonidine.

Significance to Biomedical Research and the Program of the Institute.

Numerous clinical studies have documented the hypertensive effect of chronic ethanol consumption, and an experimental model in rat has also been developed. There is some evidence that this effect is due to an impairment of baroreflex function by ethanol, but the neuronal pathways involved have not been clarified. Studying this latter problem will further our understanding of the mechanism of the cardiovascular effects of chronic ethanol consumption.

Proposed Course:

The functional integrity of the brainstem α2-adrenergic, endorphinergic and GABA-ergic systems will be studied in normal control and chronically ethanol-fed rats, measuring blood pressure, heart rate and baroreflex sensitivity as end responses.

Publications:

Mosqueda-Garcia R, Kunos G. Opiate receptors and the endorphin-mediated cardiovascular effects of clonidine in rats: evidence for hypertension-induced μ-subtype to δ-subtype changes. Proc Natl Acad Sci USA 1987 84:8637-8641.

Mosqueda-Garcia R, Kunos G. Peripheral opiate receptors are not involved in the naloxone-sensitive cardiovascular effects of clonidine in rats. Brain Res 1988 442:119-123.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00402-01 LPPS

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.)

Brainstem neuro-mechanisms and blood pressure regulation

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

	Name	Title	Affiliation
P.I.:	G. Kunos	Chief	LPPS, NIAAA
Others:	W. Laskey	Visiting Scientist	LPPS, NIAAA
	J. Mastrianni	Staff Fellow	LPPS, NIAAA
	S. Zakhari	Executive Secretary	NIAAA

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Stimulation of alpha-2 adrenergic receptors in the brainstem by endogenous catecholamines or antihypertensive drugs causes hypotension and bradycardia as well as a facilitation of the depressor baroreflex response. Opioid peptides or opiates acting in the same brain region have similar effects. Our earlier studies, confirmed by others, indicate that part of the effect of alpha2-adrenergic agonists (e.g. clonidine, alpha-methyldopa) is mediated indirectly through the local release of a beta-endorphin-like opioid. In addition to facilitation of the baroreflex by these two types of substances, there is also evidence for a GABA-mediated inhibition of the baroreflex. Furthermore, there are documented interactions between GABA and the above two neurotransmitters in central cardiovascular control, and all three agents appear to produce their effects at the level of the brainstem. Ethanol has been shown to reduce both β-endorphin and catecholamine levels in the brainstem, while its neurobehavioral effects have been linked to stimulation of the central GABA-benzodiazepine receptor complex. These observations raise the possibility that the hypertensive effects of ethanol, well established in population and clinical studies and linked to impaired baroreflex function, may be mediated by its interaction with central alpha-2, opioid and GABA-ergic mechanisms. We will test this possibility by studying the cardiovascular response to manipulation of these 3 central neurotransmitter systems in control and in chronically ethanol-treated rats.

PHS 6040 (Rev. 1/84)

Project Description:

Investigators:

G. Kunos	Chief	LPPS, NIAAA
E. Ishac	Visiting Associate	LPPS, NIAAA
M. Grojec	Visiting Associate	LPPS, NIAAA

Objectives:

The goal of the project is twofold: a) to further clarify the cellular mechanisms involved in the conversion of the adrenergic receptor response in hepatocytes in primary culture, and b) to determine whether or not acute or chronic ethanol treatment can influence this process.

Earlier studies have indicated that the conversion of the receptor response in hepatocytes involves both the activation of membrane phospholipase A2 and the subsequent generation of a cyclooxygenase product (Kunos et al., PNAS 81:6178, 1984; Ishac & Kunos, PNAS 83:53, 1986) and activation of protein kinase C (Kunos & Ishac, Biochem. Pharmac. 36:1185, 1987). There is also evidence that the conversion process requires the synthesis of protein other than the receptor protein (Kunos & Ishac, J. Cardiovasc. Pharmac. 7(Suppl. 6):587, 1985) Two specific aims related to the above findings will be pursued: 1) to demonstrate directly the culture-induced increase in membrane phospholipase A2 activity and to identify the arachidonate metabolite involved in the conversion response; b) to identify the protein factor required for the time-dependent change in receptor types to occur.

Methods:

The preparation of isolated hepatocytes and measurement of glyycogen phosphorylase activity will be done as described earlier (see above). Protein kinase C activity will be determined by measuring the transfer of 32P from [32P]ATP to histone as described by Wise et al. (BBRC, 99:407, 1981). Membrane phospholipase A2 activity against exogenous and endogenous substrate will be measured according to Burch & al. (PNAS 83:7201, 1986). In vivo treatment of rats with ethanol will be according to the protocol of Saito et al. (J. Neurochem. 48:1817, 1987).

Major Findings:

Most of the studies referred to above have been done at McGill University prior to FY88, and will not be detailed here. So far in the current fiscal year the laboratory space has been renovated, the necessary equipment has been obtained and personnel is being recruited. During the last few weeks, experimental work on these projects has started and has focussed on setting up methods to directly quantify phospholipase A2 activity against exogenous as well as endogenous substrates in plasma membranes prepared from isolated hepatocytes. Other experiments have been done to test the ability of polymyxin B, a relatively selective inhibitor of protein kinase C, to influence the conversion of the adrenoceptor mediated phosphorylase response.

Significance to Biomedical Research and the Program of the Institute:

Our knowledge of the cellular pathways involved in calcium-linked and cAMP-dependent hormone action has grown dramatically over the last few years, but the mechanism of interactions between these two major pathways are less well understood. The present project may help to elucidate the mechanism of such 'crosstalk' between different receptor pathways. Ethanol has been shown to selectively modify the interaction between α1 and VIP-receptors without affecting the individual receptor pathways (Chik et al., BBRC 147:145, 1987). The possibility that ethanol may influence interactions between α1 and β-adrenergic receptors is therefore plausible. If such an interaction can be demonstrated, it will further our knowledge about the effects of ethanol on autonomic nervous sytem activity and neurotransmitter receptor function.

Proposed Course:

Phospholipase A2 activity in plasma membranes prepared from freshly isolated hepatocytes (α1-response) and cells preincubated for 4 hours (β-response) will be quantified by measuring the release of arachidonic acid from radiolabelled phospholipid. The activity of the enzyme will be assessed against both exogenous substrate and endogenous substrate generated by preincubating the intact cells with labelled arachidonic acid. These experiments will test whether, in fact, phospholipase A2 activity is increased by prolonged in vitro incubation of cells. Arachidonic acid metabolism will be studied by preincubating 0 hr and 4 hr cells with 14C-arachidonic acid, and analyzing the released radiolabelled metabolites by thin layer chromatography and/or HPLC. The aim is to identify metabolites whose production is increased in the 4 hr cells. These metabolites will then be tested for their ability to influence the relative dominance of α and β-receptors in the phosphorylase response to catecholamines.

Experiments detailed in project #Z01 AA 00401-01 LPPS indicate that interleukin-1 as well as some other monocytic products can regulate β-adrenergic receptors in lung cells. Interleukin-1 is also produced by the hepatic Kupffer cells, which are part of the body's macrophage system. We will test the hypothesis that the protein factor known to be necessary for the time-dependent change in receptor response in hepatocytes may be interleukin-1.

Finally, we will test whether in vitro incubation of hepatocytes with various concentrations of ethanol or in vivo pretreatment of rats with ethanol could influence the reciprocal change in α and β-receptor responses in isolated hepatocytes.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00403-01 LPPS

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.)

Inverse regulation of hepatic alpha1- and beta-adrenergic receptors

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

P.I.:	G. Kunos	Chief	LPPS, NIAAA
Others:	E. Ishac	Visiting Associate	LPPS, NIAAA
	M. Grojec	Visiting Associate	LPPS, NIAAA

COOPERATING UNITS (if any)

LAB/BRANCH Laboratory of Physiologic and Pharmacologic Studies

SECTION Office of the Chief

INSTITUTE AND LOCATION NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS: 2.2	PROFESSIONAL: 2.2	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.)

There is evidence for the involvement of membrane-bound receptor and enzyme systems in the biological effects of ethanol. It is also widely assumed that ethanol or adaptive changes to ethanol consumption do not affect membrane proteins directly, but rather influence their activity indirectly through actions on membrane lipids and changes in membrane fluidity. One might expect then that interactions between different membrane-proteins would be particularly sensitive to ethanol. We have long been interested in the mechanism of interaction between calcium-linked alpha- and cyclic AMP-linked β-adrenergic receptors. We have shown that in vitro incubation of isolated rat liver cells in a serum-free medium leads to a rapid conversion of the adrenergic activation of glycogen phosphorylase from an alpha-1 to a beta-receptor mediated event. This conversion reflects inverse, reciprocal changes in the coupling of the two receptors to their respective post-receptor pathways, and requires protein synthesis. Activation of membrane phospholipase A2 and protein kinase C are also involved in the conversion response. Studies in progress are aimed to further clarify the nature of the phospholipase and protein involvement. We will also determine whether in vitro or in vivo treatment with ethanol would influence the time-dependent change in adrenergic receptor phenotype in isolated rat liver cells. These studies could add to our understanding of how ethanol influences adaptive changes in neurotransmitter receptor function.

PHS 6040 (Rev. 1/84)

Project Description

Investigators:

G. Kunos	Chief	LPPS,NIAAA
M. Virmani	Research Chemist	LPPS,NIAAA
T. Nakane	Visiting Fellow	LPPS,NIAAA
J. Oppenheim	Chief	LMI,FCRF,NCI

Objectives:

One objecvtive is to further characterize the lymphokine effect on β-adrenergic receptors by answering the following questions: a) what is the nature of the 35 and 70 kD proteins? b) what is the mechanism of the glucocorticoid potentiation of IL-1 action?
A second objective is to test whether ethanol can influence the production of β-receptor increasing activity in cultured lymphocytes. Such an effect may not be unexpected in view of a demonstrated effect of ethanol on IL-2 production and the ability of ethanol to impair β-adrenergic mechanisms in other tissues.

Methods:

A549 lung cells and IM-9 lymphocytes were cultured in RPMI 1640 medium containing 10% fetal calf serum, under standard culturing conditions. For testing the effects of various agents on β-receptor density or for harvesting LCM, cells were resuspended in serum-free Dulbecco MEM for 24 hours with or without the test substance. β-Adrenergic receptors in freeze-thawed cells were assayed by 125I-cyanopindolol binding in the presence and absence of 2 μM propranolol. LCM concentrated by ultrafiltration over an Omega 10K filter was subjected to gel permeation HPLC, calibrated by protein standards. Fractions eluted with phosphate buffered saline were assayed for β-receptor increasing activity in A549 cells.

Major Findings:

Coculturing IM9 human lymphocytes and A549 human lung adenocarcinoma cells results in a 2-3-fold increase in the density of β-adrenergic receptors in the latter, as quantified by 125I-cyanopindolol binding Lymphocyte conditioned medium (LCM) has the same effect, which is moderately sensitive to heat, is retained by ultrafiltration over a 10,00 m.w. cut-off filter, and is reduced by trypsin treatment or by preincubation of lymphocytes with cycloheximide, 0.3 μg/ml. Treatment of lung cells with cycloheximide also prevents the effect of LCM. Glucocorticoids, which also increase β-receptor density in A549 cells, markedly potentiate the effect of LCM. Gel permeation HPLC of LCM yields 3 peaks of biological activity with approximate Mr 70,000, 35,000 and 15,000. Monocytic interleukin-1 (IL-1) mimics the effect of LCM in that it increases β-receptor density in A549 cells (EC50: 0.3 pM) and its effect is potentiated by cortisol. Recombinant IL-1α is somewhat more potent than IL-1β, while interleukin-2 and interferon-α are ineffective. Tumer necrosis factor-α causes a small increase in β-receptors, which is not influenced by glucocorticoids. A polyclonal anti-IL-1 antibody inhibits the effect of IL-1 and the effect of the 15 kD but not the 35 and 70kD fractions of LCM. The activity of the latter two fractions is also unaffected by anti-TNF-α antibody. These results indicate that lymphocytes release protein factors including IL-1 that upregulate pulmonary β-adrenergic receptors by an action that involves protein synthesis.

Significance to Biomedical Research and the Program of the Institute:

β-Adrenergic hypofunction associated with a decrease in β-receptors are pathogenic features in bronchial asthma, and prenatal β-adrenergic stimulation helps to prevent respiratory distress by promoting the secretion of lung surfactant. Glucocorticoids have therapeutic value in both pathological conditions, which is due in part to their ability to upregulate β-receptors and potentiate their action in the lung. The similar and much more potent action of IL-1 and its synergistic interaction with glucocorticoids raises the possibility that IL-1-like cytokines are involved in the physiological regulation of β-receptors in the lung. One can also speculate that a deficiency in the release or cellular action of such cytokines may have a role in the pathomechanism of respiratory diseases associated with β-adrenergic hypofunction. The potential implication for the biology of ethanol may depend on whether or not ethanol can influence the release or action of such cytokines.

Proposed Course:

A primary aim of further studies is to identify the 35 and 70 kD proteins with β-receptor increasing activity. Concentrated LCM will be purified by multiple steps of HPLC (gel permeation, reverse pahse, ion exchange), and biological activity will be followed by testing the effect of fractions on β-receptors in A549 cells. Polyclonal antibodies against the purified peptides will be generated and used to screen a cDNA library from IM-9 cells.
Another aim is to determine the mechanism by which glucocorticoids potentiate the effect of IL-1 on A549 cells. Published evidence indicates that glucocorticoids can upregulate IL-1 receptors in lymphocytes (Akahoshi et al., J. exp. Med. 167:924, 1988). If such an effect can be demonstrated to occur in A549 cells, it may explain the synergistic interaction observed by us. These studies will be done in collaboration with Dr. J. Oppenheim (NCI).
Finally, we will test whether exposure of IM-9 cells to varying concentrations of ethanol can modify the amounts of IL-1-like activity released by these cells. We will also test whether exposure of A549 cells to ethanol can modify the effect of IL-1 on β-receptors.

Publications:

Stern L, Kunos G. Synergystic regulation of pulmonary β-adrenergic receptors by glucocorticoids and interleukin-1. J Biol Chem, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00479-05 LPPS

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.)
Synaptic and Neurosecretory Mechanisms and Ethanol Actions

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

P.I.:	F.F. Weight	Section Chief	LPPS, NIAAA
Others:	L.G. Aguayo	Staff Fellow	LPPS, NIAAA
	C.S. Rabe	Senior Staff Fellow	LPPS, NIAAA

COOPERATING UNITS (if any)
Dept. Physiol., Creighton U. (J.A. Wilson); Lab. Neurophysiol., NINCDS (D.L. Lewis); Howard Hughes Medical Inst., Columbia U. (P. Yavari); Dept. Neurosci., Roche Inst. Mol. Biol. (S. Korn)

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SECTION
Section of Electrophysiology

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

It is known that ethanol can affect neurotransmitter release and neurosecretion. The mechanisms involved in these effects, however, have not been established. We are studying neurosecretory mechanisms and the effects of ethanol on those mechanisms in several secretory cell types. Mammalian pineal cells have a well defined neuroendocrine function that involves the release of melatonin. We have studied membrane currents in pineal cells acutely dissociated from adult rats using the whole cell patch-clamp technique and found two distinct potassium currents, a slowly activating sustained current similar to the delayed rectifier and a transient current similar to the A current. At normal external calcium concentrations, no calcium or calcium-activated currents were observed. We studied calcium current and calcium-activated chloride current in the ACTH secreting mouse pituitary cell line, AtT-20. We found that the protein kinase C activator 1-oleoyl-2-acetylglycerol (OAG) reduced voltage-dependent calcium current, as did the phorbol esters 12-deoxyphorbol-13-isobutyrate and phorbol-12,13-diacetate. These data suggest that protein kinase C may be an inhibitory regulator of voltage-dependent calcium channels. Analysis of the calcium-activated chloride current revealed that the decay kinetics are largely due to mechanisms that regulate intracellular calcium. The relationship between intracellular calcium and neurosecretion was studied in the catecholamine secreting rat chromaffin cell line, PC12. We found that muscarine-stimulated release of catecholamine is associated with an inositol triphosphate-induced mobilization of intracellular calcium. Ethanol inhibited both the release of neurotransmitter and the increase of intracellular calcium. The significance of the project derives from the fact that the characterization of neurosecretory mechanisms and the actions of ethanol on those mechanisms should increase our understanding of the cellular basis of ethanol's actions in the nervous and endocrine systems.

PHS 6040 (Rev. 1/84)

Project Description

Investigators:

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Objectives:

Ethanol has been reported to alter synaptic transmission and neurosecretion in the nervous and endocrine system; however, the mechanisms involved in these actions are poorly understood. The objectives of this project are to characterize synaptic and neurosecretory mechanisms and the actions of ethanol on those mechanisms.

It is well known that Ca^{2+} plays an important role in synaptic transmitter release and neurosecretion. Previous studies indicate that ethanol can alter Ca^{2+} metabolism in synaptosomes (pinched off nerve terminals). This suggests that the effects of ethanol on transmitter release and neurosecretion may result from alterations in cellular Ca^{2+} metabolism. Since it is not possible, at the present time, to study ion channels or intracellular Ca^{2+} signals in nerve terminals, we have conducted experiments on three types of neurosecretory cells: (1) the mouse pituitary cell line, AtT-20; (2) the rat chromaffin cell line, PC12; and (3) cells from the rat pineal gland.

Methods Employed:

1. AtT-20 cells:

a. Calcium current:

AtT-20 cells were cultured in Dulbecco's Modified Eagle medium containing 10% fetal calf serum at 37°C in a humidified 10% CO_2 atmosphere. After subculturing 8-10 days, membrane currents were recorded using the patch-clamp method in the whole cell voltage-clamp mode. The extracellular solution contained: 150mM TEA-Cl, 0.8mM $MgCl_2$, 5.4mM KCl, 10mM $CaCl_2$, 10mM HEPES/CsOH (pH 7.4) and 1 uM tetrodotoxin with an osmolarity of 340 mosmol/kg. The intracellular patch pipette solution contained: 120mM CsCl, 11mM EGTA, 2mM TEA-Cl, 2mM $MgCl_2$, 10 HEPES/CsOH (pH 7.4), 4mM MgATP, 20mM creatine phosphate, and 50 U/ml creatine kinase with an osmolarity of 318 mosmol/kg. Cells were voltage clamped to a holding potential of -80mV and stepped to +10mV, the peak of the current-voltage relationship for calcium current. All recording was at room temperature, 20-22°C. Drugs were applied via a macropipette lowered into the recording bath near the surface of the cell under study. The macropipette was withdrawn from the bath to terminate drug application.

b. Calcim-activated chloride current:

A Ca^{++}-dependent Cl^- current has been observed in several mammalian and non-mammalian cell types. To understand the role of this current in the regulation of cell excitability in secretory cells, we have investigated the mechansims

that underly its activation and deactivation in AtT-20 mouse pituitary cells. The Ca^{++}-dependent Cl^- current can be activated in AtT-20 cells by step-depolarization into the range that activates the voltage-activated Ca^{++} current. In the studies described below, we have used the whole-cell patch clamp technique to examine the roles of voltage and Ca^{++} in the activation of the Cl^- current. The patch-clamp methods used were similar to those described above except for the external and internal solutions. The main solutions used in this study are listed in the following table.

External Solutions (mM).

	NaCl	TEA-Cl	$CaCl_2$	$MgCl_2$	HEPES	glucose
1.	150		5	2.0	10	20
2	146		2	10.0	10	20
3.	155		2	0.8	10	20
4.		156	5	2.0	10	20
5.		160	5	0.8	10	20
6.		158	2	2.0	10	20

Internal Solutions (mM)

	CsCl	Cs_2SO_4	HEPES	EGTA	BAPTA	$CaCl_2$	ATP Regenerating System*
1.	165		10	0.2			
2.	165		10				
3.	165		10	5		2.38	
4.	50	90	10				
5.	30	117	10				
6.	150		10				+
7.	130		10	5		0.42	+

Solutions were adjusted to pH 7.36 with NaOH (external solutions 1-3) or CsOH (external solutions 4-6, internal solutions 1-7). Osmolality ranged from 335-340 mOsm/kg (external) and 309-320 mOsm/kg (internal).

*The ATP regenerating system consisted of 4 mM ATP-Mg, 20 mM creatine phosphate and 50 units/ml creatine kinase.

2. PC12 cells:

The rat chromaffin cell line, PC12, has many characteristics in common with sympathetic neurons. In addition to nicotinic receptors, whose activation stimulates secretion, muscarinic binding sites have been identified on PC12 cells (Nature 297: 152, 1982). However, little is known regarding the functional significance of these muscarinic binding sites. We studied the effect of muscarinic agonists on intracellular Ca^{2+} mobilization, phosphoinositide metabolism and transmitter release from PC12 cells.

Intracellular free Ca^{2+} was measured using the fluorescent Ca^{2+} indicator Quin 2. Cells were loaded with 10 uM Quin 2 AM for 20 min, washed and resuspended at concentration of 2×10^6 cells/ml in HEPES buffered saline containing 1.8 mM Ca^{2+}. Phosphoinositide metabolism was studied by prelabeling cells with [^{3}H] inositol (5 uCi/ml) for 24 hr. To investigate neurotransmitter release, cells were preloaded for 30 min with [^{3}H]-norepinephrine (500 nM, 23.1 Ci/mmol).

3. Pineal cells:

Cells in the pineal gland secrete the hormone melatonin and it has been reported that ethanol administration affects the secretion of melatonin. To determine the membrane events associated with secretion, we first characterized the membrane currents in pineal cells. Membrane currents of dispersed pineal cells were studied using the whole-cell patch-clamp recording technique. Single cells from the pineal gland were acutely separated with enzymatic techniques from male rats (200-=300 g) kept under LD12:12 with lights on from 6 A.M. to 6 P.M. The artificial cerebrospinal fluid contained (mM): 150 NaCl; 5.4 KCl; 2 $CaCl_2$; 1 $MgCl_2$; and 10 HEPES; pH 7.4 and internal solution was composed of (mM): 130 KCl; 1 $CaCl_2$; 2 $MgCl_2$; 10 HEPES; and 11 EGTA.

Major Findings:

1. AtT-20 cells:

a. Calcium current:

AtT-20 cells were voltage-clamped at a holding potential of -80 mV and stepped from -120 to +80 mV using steps 100 ms in duration. Cell input resistance, measured between -120 and -60 mV, was 2.0-7.9 Gohm. An inward current activated rapidly at potentials positive to -40 mV, peaked in 6-7 ms, inactivated slowly, was inhibited by 2mM Co^{2+}, and was present in 10 mM Ba^{2+}. I-V curves for this voltage-dependent Ca^{2+} current (I_{Ca}) were obtained by measuring I_{Ca} at peak amplitude.

The role of protein kinase C in regulating Ca^{2+} channel activity was investigated using the whole-cell patch-clamp technique in AtT-20 cells. Extracellular application of the protein kinase C activator 1-oleoyl-2-acetyl-glycerol (OAG) reduced voltage-dependent Ca^{2+} current. This effect was reversible and dose-dependent (10-100 µM). Pertussis toxin did not block the effect of OAG on Ca^{2+} current suggesting that OAG does not affect Ca^{2+} channels via a pertussis toxin sensitive GTP-binding protein. Na^+ free solutions did not block the effect of OAG on Ca^{2+} channels suggesting that this effect of OAG does not involve the Na^+/H^+ antiporter. The phorbol esters 12-deoxyphorbol 13-isobutyrate (10µM) and phorbol 12,13-diacetate (100 µM) also reduced Ca^{2+} current. The results suggest that protein kinase C may be an inhibitory regulator of voltage-dependent Ca^{2+} channels.

b. Calcium-activated chloride current:

Voltage-clamp recordings of the Ca^{2+}-activated Cl^- current were also made from AtT-20 cells using the whole-cell patch-clamp technique. Cells were perfused internally with Cs^+ to block K^+ currents and bathed externally with either 1 µM tetrodotoxin or with tetraethylammonium (TEA) as a Na^+-substitute to block voltage-activated Na^+ currents. Depolarizing voltage steps from a holding potential of -80 mV to potentials positive to -30 mV evoked two currents: a fast inward current that activated between -30 mV and _+70 mV and a slowly-activating current (designed "slow step current") that was inward between -30 mV and near 0 mV (the Cl^- equilibrium potential), and outward positive to about 0 mV. Repolarization to -80 mV revealed a slowly decaying, inward tail current, whose magnitude with respect to step potential closely matched the current-voltage (I-V) relationship of the voltage-activated Ca^{++} current. Activation of the fast inward current, slow step current, and tail current, was prevented by extracellular application of Cd^{++} or removal of extracellular Ca^{++}. Replacement of extracellular Ca^{++} with Ba^{++} potentiated the fast inward

current but blocked the slow step and tail currents. Intracellular perfusion with greater than 1 mM of the Ca^{++} chelators EGTA or BAPTA prevented activation of the slow step and tail currents, but not the fast inward current. The reversal potential of the slow inward current was sensitive to changes in the Cl^- equilibrium potential but not to substitution of TEA for Na^+. The slow step current but not the fast inward current was partially blocked by the Cl^- channel blocker, SITS. These data indicate that both the slow inward tail current and the slowly-activating, reversible step current were a Ca^{++}-dependent Cl^- current. The fast inward current was a voltage-activated Ca^{++} current. In the absence of intracellular EGTA, the tail current decayed with complex kinetics, its timecourse apparently dependent on the magnitude of the voltage-activated Ca^{++} current. In the presence of 200 µM intracellular EGTA, the tail current decayed significantly faster and often decayed exponentially. Intracellular EGTA greatly reduced the apparent dependence of tail current duration on the magnitude of the voltage-activated Ca^{++} current. In the absence of intracellular EGTA, replacement of extracellular Na^+ with TEA or tetramethylammonium (TMA) increased the amplitude and prolonged the duration of the tail current. Intracellular perfusion with 200 µM EGTA prevented the prolongation but not the amplitude increase that followed Na^+ substitution. The timecourse of tail current decay was midly voltage-depdnent. Thus voltage-dependence remained following intracellular perfusion with 200 µM EGTA or replacement of extracellular Na^+ with TEA. The Ca^{++} dependent Cl^- current was not activated when intracellular Ca^{++} was buffered at 0.1 µM or less. With internal Ca^{++} buffered between 0.5 and 1.0 µM and Ca^{++} omitted from the external solution, the Cl^- current appeared to be activated at membrane potentials between -80 and -50 mV. Under these conditions of constant Ca^{++} concentrations, membrane depolarization resulted in additional Cl^- current activation. These data suggest that the complex decay kinetics of the Ca^{++}-activated Cl^- current are largely due to mechanisms that modulate intracellular Ca^{++} levels. At least one of these mechanisms is dependent on extracellular Na^+. The decay timecourse is also mildly voltage-sensitive, and this voltage-sensitivity appears to be independent of mechanisms that modulate intracellular Ca^{++} levels. Finally, buffering intracellular Ca^{++} at an elevated level is sufficient to activate the slow Cl^- current. Moreover, in the presence of 0.5 to 1.0 µM intracellular Ca^{++}, activation of the Ca^{++}-dependent Cl^- current is voltage-sensitive.

2. PC12 cells:

When PC12 cells were exposed to muscarine, the cells rapidly responded with elevation of cellular inositol trisphosphate levels, elevation of intracellular Ca^{2+} and release of stored transmitter. These three phenomena were totally inhibited by the muscarinic antagonist, atropine, but were unaffected by the nicotinic antagonist, d-tubocurarine. Muscarine-stimulated increases in inositol trisphosphate, intracellular Ca^{2+} and neurotransmitter release displayed similar time courses and concentration-dependencies suggesting that the secretion observed may be associated with the formation of inositol trisphosphate and elevation of intracellular Ca^{2+}. The increase in intracellular Ca^{2+} appeared to be due to a mobilization of Ca^{2+} from intracellular stores since the increase in intracellular Ca^{2+} was not inhibited by the voltage-dependent Ca^{2+} antagonist, nifedipine, at concentrations demonstrated to block K^+-induced Ca^{2+} influx into the cells, and no uptake of $^{45}Ca^{2+}$ was noted when cells were stimulated with muscarine. Elevation of inositol trisphosphate, intracellular Ca^{2+} and stimulation of transmitter release were, however, partially dependent on the presence of extracellular Ca^{2+}. The results suggest that muscarine-stimulated release of

neurotransmitter may be associated with an inositol trisphosphate-induced mobilization of intracellular Ca^{2+}.

The effect of ethanol on muscarine-stimulated release of $[H^3]$-l-norepinephrine ($[^3H]NE$) was also studied in PC12 cells. At concentrations of 25 mM and above, ethanol produced a dose-dependent inhibition of muscarine-stimulated release of $[^3H]NE$. The inhibition of muscarine-stimulated transmitter release occurred in the absence of any apparent effect of ethanol on $[^3H]NE$ uptake or an muscarinic

binding to the cells. Muscarinic stimulation also elevated intracellular free Ca^{2+} and this elevation was inhibited by ethanol. At concentrations greater than 100 mM, ethanol produced an increase in the basal release of $[^3H]NE$. Intracellular free Ca^{2+} was also increased by ethanol concentrations greater than 100 mM. The results suggest that the effects of ethanol on neurotransmitter release are associated with ethanol effects on intracellular free Ca^{2+}.

3. Pineal Cells:

Membrane currents of dispersed pineal cells were studied using the whole-cell patch clamp recording technique. The macroscopic ionic current observed in artificial cerebrospinal fluid was dominated by an outward current component with little or no apparent inward current. Study of the outward component in solution without added Ca^{2+} and Cl^- revealed the existence of two distinct outward currents. Depolarizations from a holding potential of -100 mV activated a fast current which reached a peak within 15 ms and completely decayed in about 150 ms. This current activated at potentials more positive than -50 mV and displayed steady state inactivation at depolarizing voltages with half-inactivation near -80 mV. The second outward current isolated from a holding potential of -50 mV activated at potentials positive to -20 mV, reached a steady state current amplitude within 50 ms and was sustained up to 400 ms. In the presence of 2 mM external Ca^{2+}, the I-V relationship did not display a region of negative slope conductance (N-shape) suggesting that Ca^{2+}-activated K^+ current did not contribute significantly to the outward current. In solutions designed to isolate calcium currents, a small inward current (<100 pA) was observed. It had a threshold at -40 mV and its amplitude reached a peak at about -10 mV. We conclude that acutely dissociated pineal cells display two distinct K currents: (i) a transient current similar to the A current (I_A); and (ii) a slowly activating, sustained current similar to the delayed rectifier current (I_K).

The effect of ethanol on these neurosecretory mechanisms is currently being investigated.

Significance to Biomedical Research and the Program of the Institute:

The cellular mechanisms involved in ethanol's actions in nervous and neuroendocrine tissues are poorly understood. Characterization of synaptic and neurosecretory mechanisms and the effects of ethanol on those mechanisms holds the promise of increasing our understanding of the cellular basis of ethanol's actions in these tissues.

Proposed Course:

Synaptic and neurosecretory mechanisms will be characterized more fully, and the actions of ethanol on those mechanisms will be investigated more

extensively. In addition, the actions of other alcohols and CNS depressantssuch as barbiturates, opiates and benzodiazepines will be characterized and compared to ethanol.

Publications:

Aguayo LG. Characterization of outward currents in a neurosecretory cell acutely isolated from the adult rat. Adv Alc Subst Abuse, in press.

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Lewis DL, Weight FF. The protein kinase C activator 1-oleoyl-2-acetylglycerol (OAG) inhibits voltage-dependent Ca^{2+} current in the pituitary cell line, AtT-20. Neuroendocrinology 1988; 47: 169-175.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00480-05 LPPS

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.)

Nerve Cell Excitability and Ethanol Actions

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

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	

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.)

Ethanol is known to affect the excitability of the nervous system; however, the cellular mechanisms underlying such actions are poorly understood. The objectives of this project are to characterize the mechanisms regulating nerve cell excitability and the effects of ethanol on those mechanisms. Electrophysiological methods were used to characterize the membrane mechanisms that underlie excitable phenomena. The membrane ion currents that are involved in the regulation of neuronal excitability were investigated in neurons from superior cervical and nodose ganglia using the whole-cell patch-clamp technique, in striatal neurons using single-channel recording and in hippocampal pyramidal neurons using the single-electrode voltage-clamp method. Two different sodium currents were characterized in the neurons from nodose ganglion: tetrodotoxin (TTX)-sensitive and TTX-resistant. The nodose neurons also have two calcium currents, transient and sustained. By contrast, the sympathetic neurons have only a TTX-sensitive sodium current and a sustained calcium current. At least three different potassium currents have been characterized in the sympathetic neurons: voltage-activated transient, delayed rectifier, and sustained calcium-activated. Similar potassium currents have been characterized in hippocampal CA3 pyramidal neurons. Studies of transmitter regulation of these currents has revealed that the sustained calcium-activated potassium current in hippocampal neurons is inhibited by muscarinic receptor activation, single potassium channels are opened by dopamine in striatal neurons, and the calcium current in sympathetic neurons is inhibited by somatostatin. The effect of ethanol on these currents is currently being investigated. The significance of the project lies in the fact that the identification of the mechanisms involved in nerve cell excitability and the investigation of the action of ethanol on those mechanisms holds the promise of increasing our understanding of the cellular basis of ethanol's actions in the nervous system.

Project Description

Investigators

F.F. Weight	Section Chief	LPPS, NIAAA
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Objectives:

Although it is well known that the administration of ethanol can affect nervous system excitability, the cellular basis of such actions is poorly understood. The objectives of this project are to characterize the mechanisms regulating nerve cell excitability and the effects of ethanol on those mechanisms.

Methods Employed:

Excitability mechanisms were characterized by three electrophysiological methods: (1) whole-cell patch clamp recording of acutely isolated adult rat nodose or superior cervical ganglion cells; (2) single-channel recording from neurons acutely isolated from corpus striatum; and (3) single-electrode voltage-clamp recording of CA3 pyramidal neurons in hippocampal slice.

1. Whole cell patch-clamp experiments on nodose and sympathetic ganglion cells:

Cell isolation procedure: Single nodose or superior cervical ganglion somata were acutely isolated by enzymatic dispersion from male and female Sprague Dawley rats (100 - 300 g). The rats were decapitated with a laboratory guillotine and the heads placed in iced Hank's balanced salt solution (HBSS). The ganglia were removed and placed modified HBSS supplemented with 6.5 g/l glucose and 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.4. Each ganglion was cleaned of connective tissue and minced with fine iridectomy scissors. The tissue fragments were then transferred to 5 ml of modified HBSS containing 1 mg/ml trypsin (type III), 1 mg/ml collagenase (type IA), and 0.1 mg/ml DNAase (type III). The tissue fragments were incubated for 1 hr at 35°C in a 25 cm^2 tissue culture flask which allowed microscopic inspection of the tissue fragments. After incubation, the flask was shaken vigourously which released the cell somata from the ganglion fragments. The enzyme solution containing the dispersed cells was then inhibited by the addition of 7 ml of modified HBSS containing 2 mg/ml soya bean trypsin inhibitor (type II-S), 1 mg/ml bovine serum albumin (BSA), 10% fetal calf serum (Gibco), and 5 mM $CaCl_2$. The cell suspension was then added to 35 mm poly-L-lysine coated tissue culture dishes and superfused with physiological saline. The enzymes and inhibitor were obtained from Sigma Chemical Co. St. Louis, Mo. and prepared daily.

Patch-clamp recording: Microelectrodes were fabricated from borosilicate glass capillaries (1.69 mm od, 1.35 mm id; Drummond Scientific Co., Broomall, Pa.) using a Narashigi PW-6M microelectrode puller, coated with Sylgard (Dow Corning Corp., Midland, MI) to reduce the electrode capacitance and the tip-fire

polished on a microforge. Pipettes filled with internal solutions had resistances in the range 0.3-2 Mohms. The cells were voltage-clamped using a L/M-EPC7 patch clamp amplifier (List Electronic, West Germany). Patch electrodes were sealed against the membrane by suction yielding seal resistances greater than 10 Gohms. A holding potential of -50 mV was applied to the pipette and the electrode capacitance neutralized. The membrane patch was then disrupted by further suction after which the membrane capacitance and series resistance controls were optimally adjusted. The cells were then clamped at the selected holding potential and a series of hyperpolarizing and depolarizing command potentials were delivered. Membrane currents were filtered using a 4 pole Bessel filter (3KHz - 3dB) (Krohn-Hite 3750), digitized with a 12 bit A/D converter, and stored for analysis using a PDP-11/23 microcomputer. Records were digitized at 100 usec/point (Na^+ currents) or 150 usec/point (Ca^{++} currents). Current traces and current/voltage relationships were corrected for linear leakage current measured from hyperpolarizing command pulses. Test solutions were applied by lowering a large bore (>10 um) pipette, containing the test solution, close to the cell soma. All experiments were conducted at 19 to 23°C.

2. Single-channel experiments on striatal neurons:

Cell isolation procedure: Male Sprague-Dawley rats, 31-45 days post partum, were the tissue source. The corpus striatum was rapidly and carefully dissected in a beeswax-coated Petri dish under ice-cold Pipes saline [120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM D-glucose, 20 mM Pipes NaOH (pH 7.0 at 25°C, preequilibrated with 100% O_2)] and hand-minced with a scalpel blade into blocks of ~ 2 mm per edge. The tissue blocks were transferred by using a wide-bore pipette to a 25-ml jacketed double-side-arm Wheaton Celstir stirring chamber, containing 130,000 benzoylarginine ethyl ester units of trypsin (Sigma type XI) in 12 ml of Pipes saline. The tissue was stirred at ~ 40 rpm under 100% O_2 at 32°C, with the stirring rate adjusted so that the tissue blocks were raised above the bottom of the chamber but not so fast as to cause the solution to become cloudy. The incubation was continued for 1 hr, after which the solution was replaced with fresh saline and trypsin, and the incubation was continued for a second hour. Soybean trypsin inhibitor (Sigma type 1-S; 7.8 mg in 0.5 ml of Pipes saline) was then added. After 5 min, the solution in the chamber was removed, and the tissue was washed twice with 12.5 ml of Dulbecco's modified Eagle's medium (DMEM) [bicarbonate-free, with 25 mM Hepes, 25 mM glucose, 4 mM glutamine (adjusted with NaOH to pH 7.4 at 25°C, and with NaCl to 340 mosmol/kg)]. A third volume of DMEM was added, and incubation was continued at 32°C for up to 6 hr. As needed, 3 or 4 tissue blocks were transferred with ~ 0.25 ml of DMEM to a 1.5-ml conical tube and tapped by finger until the solution became cloudy and the tissue blocks were reduced in size. The supernatant was poured into a polyornithine-coated Petri dish, and the cells were allowed to settle for 10 min before beginning electrophysiological recording, which was performed within 1 hr.

Single-channel recording: The cells were continuously superfused at room temperature in a solution containing 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 10 mM Hepes NaOH (pH 7.4, adjusted with sucrose to 340 mosmol/kg). Borosilicate glass patch pipettes were filled with the intracellular solutions and had resistances to 3-5 Mohms. Patch-clamp recording was performed in the whole-cell and cell-attached configurations by using a List EPC-7 amplifier. Amplifier output went through an 8-pole Bessel filter (4-kHz lowpass for whole-cell recording or 2-kHz lowpass for cell attached recording) to a 12-bit A/D converter (5 kHz for whole-cell voltage-

clamp, 8 kHz for current-clamp, and 10 kHz for single-channel recordings) for storage and analysis on a PDP-11/73 computer. Recordings were made from phase-bright cells free of apparent membrane blisters and retaining visible neuritic processes. Whole-cell recordings were deemed suitable for use if membrane potential was not less negative than -40 mV and if the action potential amplitude exceeded 80 mV. Cell-attached recordings were used if seal resistance exceeded 5 Gomega and if the cell remained phase-bright throughout the recording.

3. Single-electrode voltage-clamp experiments on CA3 pyramidal neurons:

Brain slice preparation: Slices were prepared from the hippocampus of male Hartley guinea pigs (300-600 g). The hippocampus was cooled by immersion in iced artificial cerebrospinal fluid (CSF) and slices (400-450 um) were prepared on a drop-blade tissue chopper (Sorval). The slices were immediately placed in a prechamber for later use. The prechamber was designed to maintain slices at room temperature in constantly circulating artificial CSF that had been equilibrated with 95% O_2-5% CO_2. Slices were kept in the prechamber for a minimum of 1 h, allowing the tissue to stabilize before recording was performed. A single slice was then positioned in a recording chamber and held submerged in the artificial CSF equilibrated with a gas mixture of 95% O_2-5% CO_2.

Solutions: The artificial CSF used had the following composition (mM): NaCl 124, CKl 3.2, $CaCl_2$ 2.4, $MgCl_2$ 1.3, $NaHCO_3$ 26, NaH_2PO_4 1.2, glucose 10. The pH was 7.4 after bubbling with 95% O_2-5% CO_2. Tetrodotoxin (TTX, 2 x 10^{-7} M) was present in all experiments to prevent sodium spikes from occurring during depolarizing steps. In experiments in which $MnCl_2$ or $CdCl_2$ was added to the solution, NaH_2PO_4 and $MgSO_4$ were omitted. When studying the effects of Mn^{2+} or Cd^{2+}, $MgCl_2$ was added to the control solution to keep the divalent ion concentration constant throughout the experiment

Voltage-clamp: A single-microelectrode voltage/current clamp (SEC) based on the design of Wilson and Goldner was used for intracellular potential measurements and voltage clamping of the neuronal membrane. The switching frequency of the clamp was set at 4-5 kHz with current passed during 25% of the cycle. We used relatively low resistance microelectrodes fabricated on a Brown-Flaming microelectrode puller using thin-walled borosilicate tubing (ID 0.9, OD 1.2 mm; Frederick Haer). Such microelectrodes typically had resistances of 15-30 Mohms when filled with 3 M CsCl and had tip lengths (i.e., the distance from the initial narrowing of the glass to the tip) of 8-12 mm. In comparison with higher-resistance microelectrodes, these microelectrodes allowed more current to be passed and allowed the clamp to be operated at a higher gain, reducing discrepancies between the command potential and actual potential of the cell.

Data acquisition: The SEC output was connected to an LSI-11/23 microcomputer system, and data was digitized and stored on magnetic media for subsequent analysis. The current signal was first passed through a low-pass Butterworth filter (24 dB/decade) with the corner frequency set at 300 Hz for 1-s voltage steps and at 750 Hz when observing faster events during 100-ms steps. The microcomputer was also used to generate the voltage commands.

Major Findings:

1. Whole cell patch-clamp recording of nodose and sympathetic ganglion cells: The electrical properties of nodose ganglion cells acutely isolated from adult rats were studied using the whole cell patch-clamp recording method. Current-clamp recording revealed a mean resting membrane potential of -54.3 mV and an input resistance of 527 Mohms. Depolarizing current steps evoked action potentials with the following properties (mean): amplitude 111 mV, threshold -36mV, and rate of rise 117 V/sec. Two types of action potentials were observed, short duration and long duration. These properties, with the exception of input resistance (527 Mohms cf. 50 Mohms), are similar to those reported previously using intracellular recording methods in intact nodose ganglia. Brief application of 10 uM 5-hydroxytryptamine (5-HT) resulted in a rapid depolarization and burst of action potentials in the majority of cells. With voltage-clamp recording, step depolarizations to potentials positive to -15 mV elicited a transient inward current which was followed by a sustained outward current. Inward Na^+ current was isolated by ion substitution and pharmacological agents. Two types of Na^+ current were observed. One current was completely abolished by 3 to 15 uM tetrodotoxin (TTX), had a rapid time course, activated over the potential range -70 to -10 mV and attained half-maximal conductance at -30 mV. The other current persisted in the presence of 15 uM TTX, had a slower time course, activated over the potential range -30 to 0 mV, and attained half-maximal conductance at -20 mV. In addition, 500 uM Cd^{++} and 5.0 mM Co^{++} (5 mM) reduced the maximal inward current to 5% and 20% of control respectively. When Ba^{++} was substituted for Ca^{++} as the charge carrier, the maximal inward current increased to 175% of control. Some cells had two Ca^{++} current components, an inactivating component which activated near -60 mV and a large sustained current which activated near -40 mV. The initial inactivating current appeared as a "hump" on the I/V curve over the potential range -60 to -30 mV. The results indicate that some cells have more than one type of Na^+ and/or Ca^{++} channels.

Monovalent cation selectivity and divalent cation sensitivity of the tetrodotoxin (TTX)-resistant Na^+ current in dissociated adult rat nodose ganglion neurones were investigated using the whole-cell patch-clamp technique. The TTX-resistance Na^+ current was isolated using ion substitution and pharmacological agents. Under these conditions, the current reversal potential shifted 52 mV per tenfold change in external $[Na^+]$. Inorganic and organic monovalent cation permeability ratios (P_x/P_{na}) were determined from changes in reversal potential and the Goldman-Hodgkin-Katz equation. The P_x/P_{na} values determined by the former method were $HONH_3^+$, 1.38; Li^+, 1.00;H_2NNH_3, 0.66; NH_4^+, 0.28: $CH_3NH_3^+$, <0.13; K^+, <0.13; R^+, <0.12; Cs^+, <0.10; $(CH_3)_4N^+$. <0.10. The values determined by either method agreed within 10%. The effects of Cd^{2+}, Co^{2+}, Mn^{2+} and Ni^{2+} on the TTX-resistant Na^+ current were analysed from peak-conductance values. These ions shifted the activation of the current to more positive potentials and decreased the maximal conductance. At 3 mm concentrations, Cd^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} decreased the maximal conductance 64.6, 50.7, 25.0 and 20.3% respectively. The results indicate that: (a) the monovalent cation selectivity of the TTX-resistant Na^+ current is similar to that of the TTX-sensitive Na^+ current in other tissues; and (b) the TTX-resistant Na^+ current is less sensitive to divalent cations than the Ca^{2+} current in these neurones. These observations suggest that the structure determining the monovalent cation permeability of the TTX-resistant Na^+ current is similar to that of the TTX-sensitive Na^+ current in other tissues, and that the channels carrying the TTX-resistant Na^+ current are distinct from those responsible for the Ca^{2+} current.

Potassium currents were investigated in superior cervical ganglion cells in solutions designed to isolate K^+ current. Depolarizations from a holding potential of -80mV elicited two distinct outward current components. An initial component activated at potentials more positive than -60 mV, rose rapidly, and then decayed. A second sustained current component was observed at potentials more positive than -20 mV. The initial transient current was inactivated by holding the membrane potential at -50 mV, whereas the second current component was reduced only slightly at this holding potential. The transient current decayed with a time constant of approximately 20 msec. When the holding potential was -50mMV, the transient current could be fully reactivated by a conditioning prepulse to -100mV. The time constant of reactivation at -100 mV was approximately 36 msec. This current was not blocked by 0.5 mM Cd^{2+}-containing or Ca^{2+}-free external solutions, but could be markedly reduced by 2 mM 4-amino-pyridine. The transient current had the characteristics of I_A. The sustained outward current could be further separated into two outward current components. Depolarizations from -50 mV elicited a current that activated at potentials more positive than -20 mV, and was blocked by superfusion with a 0.5 mM Cd^{2+}-containing or Ca^{2+}-free external solution. Digital subtraction revealed that the Ca^{2+}-sensitive current activated with an exponential time constant of 20 to 40 msec over the potential range 0 to +10 mV. The current that remained in the Ca^{+2}+-free external solution also activated at potentials more positive than -20 mV and was reduced by superfusion with 10 mM TEA. It is concluded that under the conditions of these experiments there appear to be at least 3 outward currents: (i) a transient K^+ current with characteristics similar to I_A; (ii) a sustained Ca^{2+}-sensitive K^+ current similar to $I_{K(Ca)}$; and (iii) a sustained Ca-insensitive, TEA-sensitive K^+ current that appears to be similar to the delayed rectifier or I_K.

Somatostatin-like immunoreactivity has been reported to occur in the postganglionic neurons of sympathetic ganglia. We have investigated the effect of somatostatin (SOM) on the Ca^{2+} current in sympathetic neurons. Voltage-clamp recordings, using the whole-cell patch-clamp technique, were made from acutely isolated adult rat superior cervical ganglion (SCG) neurons in solutions (external and internal) designed to isolated Ca^{2+} currents. Application of 0.001-1.0 μM [D-Trp^8] SOM resulted in a rapid, reversible and concentration-dependent decrease in the amplitude of the Ca^{2+} current evoked from a holding potential of -80 mV. The concentration-response relationship for SOM could be fitted to a single-site binding model with an apparent dissociation constant of 121 nM; the maximal attainable block of Ca^{2+} current by SOM was 50%. SOM also produced a pronounced slowing of the Ca^{2+} current rising phase, especially at more depolarized potentials. At higher concentrations (0.03 - 1.0 μM), prolonged application of SOM resulted in a progressive decrease in blocking ability. The results are consistent with a neurotransmitter and/or neuromodulator role for SOM in the sympathetic nervous system.

2. <u>Single-channel recording from striatal neurons</u>: Neurons acutely dissociated from the corpus striatum of young adult rats had membrane surfaces suitable for Gohm-seal recording. Whole-cell current-clamp and voltage-clamp recordings indicated that the cells remained electrically excitable after dissociation. Cell-attached recordings frequently revealed single-channel openings in the presence of dopamine or of the D_2 dopamine agonist quinpirole. Channel openings were rarely or never observed in the absence of drugs or in the presence of quinpirole plus the dopamine antagonist haloperidol. The D_2 antagonist spiperone was more potent at blocking the appearance of the channel

than was the D_1 antagonist SCH-23390. The channel reversal potential varied with the extracellular K^+ concentration as predicted by the Nernst equation. The channel current-voltage relationship was linear, with a conductance of ~85 pS in the presence of 140 mM KCl. These results are consistent with the opening of single K^+ channels following D_2 dopamine receptor activation.

3. Single-electrode voltage-clamp recording of CA3 pyramidal neurons: Inward currents in hippocampal CA3 pyramidal neurons were studied using the single microelectrode voltage-clamp (SEC) technique. Neurons in the pyramidal layer of region CA3 were impaled with a single microelectrode containing 3M CsCl. Diffusion of Cs^+ into the neurons produced a large reduction in outward K^+ currents, revealing a depolarization activated inward current. This current could be reduced by the application of Co^{2+}, Mn^{2+}, or Ca^{2+}-free solutions, and is therefore presumed to be mediated by Ca^{2+}. In Cs^+ loaded neurons the inward current activated rapidly (<30 msec) and gradually decayed with maintained depolarization. This decline in inward current was incomplete, however, and the recorded current often remained net inward during a depolarization lasting several seconds. The data indicate that the inward Ca^{2+} current rapidly activates and that a fraction of this current inactivates with maintained depolarization. This inactivation may not be dependent on Ca^{2+} since it occurred when Ba^{2+} was substituted for Ca^{2+} in the artificial CSF used. The deactivation time course for the inward current suggests that two or more types of Ca^{2+} channels are present in these cells.

Outward currents were also studied in Cs-loaded hippocampal CA3 pyramidal neurons. Several outward currents that have been previously observed in these neurons were significantly reduced or blocked by Cs^+ loading. These include a transient Ca^{2+}-sensitive current, a transient voltage-activated current, and a current similar to the delayed rectifier. Step depolarizations to potentials positive to -14 mV elicited a slowly developing outward current. The slowly developing outward current was greatly reduced or blocked by the application of 3 mM Co^{2+}, 2-4 mM Mn^{2+}, or Ca^{2+}-free external solution. The slowly developing outward current was also reduced by the application or 10-25 mM tetraethylammonium (TEA) or 0.2-1 mM Ba^{2+}. Application of 500 uM 4-aminopyridine (4-AP) had no apparent effect on this current. As the holding potential was made more positive over a range of -60 to -20 mV, the amplitude of this outward current decreased. Application of muscarine or carbachol reversibly depressed the slow outward current. However, in Ca^{2+}-free solution containing Mn^{2+} or Co^{2+}, an effect of muscarine or carbachol on outward current was not observed. The results suggest that the slowly developing outward current is a Ca^{2+}-dependent current that is inhibited by activation of muscarinic receptors. The reduction of outward current by muscarinic receptor activation may result from the reduction of Ca^{2+} current that was also observed in response to muscarinic agonists.

The effect of ethanol on the above ion currents is currently being studied.

Significance to Biomedical Research and the Program of the Institute:

The behavioral effects of ethanol and the development of dependence and tolerance to ethanol are due to the actions of ethanol on the nervous system. The cellular basis of such actions, however, is poorly understood. Characterization of the cellular mechanisms that regulate nerve cell excitability and the actions of ethanol on those mechanisms holds the promise of increasing our understanding of the cellular basis of ethanol's actions in the nervous system.

Proposed Course:

The mechanisms regulating nerve cell excitability will be characterized more fully, and the actions of ethanol on those mechanisms will be investigated more extensively. In addition, the actions of other alcohols and CNS depressants such as barbiturates, opiates, and benzodiazapines will be characterized and compared to ethanol.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00404-01 LPPS

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 calcium and phosphorylation-regulated signalling pathways

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

PI:	R.L. Kincaid	Section Chief	LPPS, NIAAA
Others:	T.M. Martensen	Research Chemist	LPPS, NIAAA
	J. Tamura	Visiting Fellow	LPPS, NIAAA

COOPERATING UNITS (if any) Penn State Univ. (M.L. Billingsley, C.D. Balaban); Lab of Immunology, NIAID, NIH (M. Sitkovsky); Univ. of Rome (R. Geremia) Molec. Neurogenetics Branch, NIMH, ADAMHA, (B. Martin)

LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

SECTION

Immunology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Ave., Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.1	0.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Investigations on the physiology and molecular biology of calcium and phosphoprotein-regulated signalling pathways have been initiated, with emphasis on the calmodulin (CaM)-dependent phosphatase, calcineurin (CN), and cyclic nucleotide phosphodiesterase (PDE). Immunocytochemical studies indicate that the calmodulin-dependent isoform of brain PDE is expressed predominantly in regional output neurons, consistent with a role in integration of synaptic input. Further, chemical destruction of climbing fiber afferents to cerebellar Purkinje cells causes loss of PDE immunoreactivity suggesting a "transsynaptic" model for regulation of gene expression. Current studies on the cloning of this enzyme are designed to determine the control mechanism(s) for expression. Recent studies have shown that CN is the major cytosolic CaM-binding protein (BP) in lymphocytes and that it is differentially expressed in subpopulations of lymphoid cells. To examine its regulation in the immune system (and brain), cDNAs for the catalytic subunit of this phosphatase have been cloned using expression vector immunoscreening and a novel plaque hybridization method employing biotinylated restriction fragments. Tissue-specific mRNA species are now being characterized and in vitro mutagenesis will be carried out to examine important allosteric and catalytic domains on the enzyme, some of which are suggested by clear similarities to other recently cloned phosphatases. Ongoing studies with model phosphopeptides will be used to investigate immunologically the phosphorylation of receptors and proteins involved in control of intermediary metabolism and cytoskeletal organization.

PHS 6040 (Rev. 1/84)

Project Description

Objectives: Calcium is known to mediate many of the intracellular responses elicited by effector or hormone stimulation. Cyclic nucleotides and phosphoproteins are also intimately involved in such "signalling pathways" and are linked to Ca^{2+} via the calcium binding protein, calmodulin (CaM). The primary goals of this project are 1) to characterize several of the CaM-dependent enzymes using immunological and molecular biological approaches, 2) to determine the regulation of and interactions between CaM-, phosphoprotein- and cyclic nucleotide-dependent pathways during activation of neural and lymphoid cells, and 3) to investigate the involvement of these signalling events in the adaptive and pathologic cellular responses to alcohol and to infective agents such as AIDS.

Methods Employed:

Purified IgG fractions from rabbit (for PDE immunocytochemistry) were made using conventional Protein-A Sepharose methods. Affinity-purified antibodies to PDE and CN were prepared by chromatography of crude antiserum on columns of antigen immobilized to CNBr-activated Sepharose, followed by elution with denaturing buffer (50 mM sodium acetate, pH 4.8, containing 6M urea, 500mM NaCl and 10 mM EDTA). Eluted antibodies were immediately dialyzed versus 50 mM Tris HCl, pH 7.5,/ 150 mM NaCl (TBS) containing 40% glycerol to remove urea. Immunocytochemistry was carried out using 70µ sections of adult rat brain that had been perfused with 10% formaldehyde. After incubation with primary antibodies to PDE or CN, the complexes were detected with peroxidase-conjugated second antibodies and visualized using diaminobenzidine and hydrogen peroxide.

Studies of the tissue distribution of CaMBPs were carried out using Western blot procedures with the above antibodies and/or biotinylated CaM, prepared as previously described. After electroblotting at a constant voltage of 100V, the nitrocellulose membrane containing immobilized proteins was incubated with primary antibody for 1-2 hr at room temperature followed by alkaline-phosphatase conjugated second antibody (or streptavidin) for 30-60 min; reactive complexes were visualized with chromogenic substrates, nitro blue tetrazolium (NBT) and brom-chloro-indolyl phosphate (BCIP).

Cloning of a cDNA encoding the CaM-binding domain of CN was carried out using a mouse brain lambda GT-11 library with conventional expression vector immunoscreening methodology.

Full length clones were isolated using a novel procedure that employs plaque hybridization to biotinylated restriction fragments; these full length cDNAs were subcloned into pUC-18 for expression of the recombinant protein and DNA sequencing using the dideoxy nucleotide termination procedure. Analysis of the DNA for sequence homologies was carried out using Gen-Bank with relational data bases prepared by commercial vendors (Bio-Rad).

Major Findings:

1) Regulation of cyclic nucleotide phosphodiesterase in brain and testis. Prior studies with Drs. Balaban and Billingsley demonstrated that in contrast to the ubiquitous distribution of CN in central neurons, PDE was selectively localized in the major output neurons of cingulate cortex, hippocampus and cerebellum and was present in high concentrations (1-10μM) in the dendrites of these cells. These data suggested a role for Ca^{2+}-regulated cyclic nucleotide metabolism in integration of synaptic input. To test this hypothesis in the cerebellum, the primary excitatory input to Purkinje cells (climbing fibers) was ablated chemically using the neurotoxin 3-acetyl pyridine (3-AP); this procedure destroys the inferior olivary nucleus where climbing fibers originate. Concomitant with the degeneration of these inputs, there was a dramatic loss of PDE immunoreactivity without a substantial change in that seen for CN or for CaM-binding proteins in general. PDE immunoreactivity in other parts of the brain was not reduced, suggesting that this change was directly attributable to the localized loss of innervation. Such a specific, lesion-dependent down regulation suggests that maintenance of high enzyme concentration in the neuron requires synaptic activation. This apparent "transsynaptic" regulation of gene expression may provide a useful model for certain aspects of developmental neurobiology and will be the subject of future studies using cDNA probes for PDE (cloning studies in progress).

In collaboration with the laboratory of Dr. Geremia, a novel isozymic form of CaM-dependent PDE was characterized in mouse testicular germ cells. The enzyme exhibited micromolar affinity for cAMP, as opposed to that usually seen for the CaM-regulated form (2μM for cGMP, 40μM for cAMP) in other tissues (brain, heart). Interestingly, adjacent cell types in the testis (Sertoli cells) contained exclusively the lower affinity form of the enzyme, suggesting cell-specific gene regulation within the testis. The high affinity cAMP isoform appears to differ from the brain enzyme in that it is a highly assymetric monomer that associates to form an $\alpha_2\beta_2$ tetramer with CaM and that it is inhibited non-competitively by substrate. Immunologically, the enzyme cross-reacted with antibodies prepared against the brain isoform, having several peptides of similar size. Thus, the germ-cell isozyme appears to

represent a form of PDE that is ancestrally related to the brain form but whose biochemical properties have been altered to permit increased specificity for cAMP.

2) Regulated expression of CN in lymphocyte subpopulations. Previous studies with Dr. Sitkovsky established that the predominant cytosolic CaMBP in murine lymphocytes was a 59kDa protein immunologically related to the brain protein phosphatase, CN; this suggested that regulation of certain phosphoproteins may be an important function during Ca^{2+}-dependent cell activation. In an extension of those studies, the amount of this enzyme has been estimated immunologically in various subsets of lymphoid cells (macrophages, B-cells, T-cells, thymocytes).

While macrophages and T-cells appeared to have similar amounts of the protein, B-cells were found to have approximately 4 times as much; interestingly, in thymocytes a CaMBP of 65 kDa, that was not immunoreactive with CN antibody was observed. However, upon limited proteolysis with Stapylococcal V-8 protease, the thymocyte-specific protein showed extensive identities with peptides produced from the splenic lymphocyte protein. These data indicated that the amount of the phosphatase differed in subpopulations of lymphocytes and that an apparent precursor form of this enzyme was present in thymocytes. The molecular basis for this differential expression of enzyme in related lymphoid cells has not been determined.

3) Molecular cloning of cDNAs for mouse brain calcineurin. In an attempt to elucidate that mechanisms involved in selective expression of protein phosphatase, cloning of this enzyme was carried out in a lambda GT-11 expression library. One clone (CNα-1) was isolated that produced an immunoreactive fusion protein that was capable of interacting with CaM. Indeed, CaM-Sepharose affinity chromatography of lysogen extracts yielded milligram quantities of a 150 kDa fusion protein (β-galactosidase portion =115 kDa). Limited digestion of this chimeric protein with protease followed by antibody detection of electroblotted fragments ("immunofingerprinting") revealed virtual identities with fragments obtained from authentic brain calcineurin. The cDNA insert (1.1 kb) was subcloned into pUC-19 and sequenced. The deduced amino acid sequence had an open reading frame coding for a protein of 34 kDa and contained seven regions corresponding to residues sequenced from bovine brain CN, providing absolute confirmation of the identity of the clone. A putative CaM-binding site was found about 100 residues from the COOH terminus that exhibited the characteristic hydrophobic and cationic properties of the CaM interaction domain.

In the process of cloning full-length cDNAs for the catalytic subunit a novel, non-radioactive plaque hybridization method was

developed. A 235 bp region on the 5′ end of the CNα-1 insert was excised from the plasmid using KpnI restriction, purified electrophoretically and biotinylated enzymatically to make a probe. Using protease digestion to reduce background color on filters and streptavidin alkaline phosphatase to detect the biotin-containing hybrids, the phage library was screened and several clones with inserts of 1.8-2 kb were isolated. One clone (CNα-4) contained the ATG initiation codon, coded for a protein of 58.3 kDa and was expressed in pUC-18. Analysis of the deduced protein sequence demonstrated two regions of high similarity to other recently cloned protein phosphatases that are unrelated in terms of substrate specificity and regulation by allosteric effectors. These findings support the view of a true superfamily of protein phosphatses and suggest that these conserved areas may correspond to the ancestral catalytic and/or regulatory domains. Studies are in progress to determine the function of these regions and to develope consensus probes against these domains.

Significance to Biomedical Research and the Program of the Institute:

The biochemical responses to hormones, growth factors and other effectors are mediated by a variety of signalling pathways, some of which interact to influence each other. Several elements that play a role in signal transduction include Ca^{2+}/CaM- dependent enzyme activation, cyclic nucleotide "second messengers" systems and modulation of phosphoprotein metabolism. Investigation of the properties, distribution and regulation of these provides important information regarding normal and pathologic cellular activity and may lead to the design of rational pharmacologic therapies. Since chronic pertubation of the cellular environment resulting from drug or alcohol exposure may well lead to changes in signalling pathways, these studies may reveal fundamental features of pharmacologic dependence, tolerance and withdrawal.

Proposed Course:

To investigate the molecular biology of enzymes involved in Ca^{2+}-dependent signal transduction (PDE,CN) and to characterize their regulation during cellular activation and differentiation. To utilize specific immunological probes prepared against important phosphorylated sites, in order to understand the regulation of immunomodulatory and growth factor receptors as well as control of intermediary metabolism. To examine signalling events under pathologic conditions (e.g. using murine models for alcoholism, inbred neuropathologies and immune disorders such as AIDS).

Publications:

Vaughan M, Kincaid, RL. Mechanism of activation of cyclicnucleotide phosphodiesterase by calmodulin: Utilization of novelbiologically active derivatives of calmodulin to probe mechanisms of Ca^{2+}-dependent interaction and enzyme activation. VI US-USSR Symposium on Myocardial Infarction, New York, Gordon and Breach Publishers, 1987, 13-29.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00472-06 LPPS

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.)
Ethanol Effects on the Immune System

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

P.I.:	C. Marietta	Physiologist	LPPS, NIAAA
Others:	F. Weight	Section Chief	LPPS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA

COOPERATING UNITS (if any)
Dept. of Pathology, University of Texas Health Science Center, Galveston, TX; (T.R. Jerrells).

LAB/BRANCH
Laboratory of Physiologic and Pharmacologic Studies

SECTION
Section on Immunology

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS: 0.7	PROFESSIONAL: 0.1	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.)

Alcoholics are known to be very susceptible to infections. We studied the effects of alcohol on the immune system of rats and mice using various models to produce physical dependence. Both normal and adrenalectomized animals were studied with respect to cell numbers and cell function. Ethanol administration resulted in the loss of lymphocytes from the peripheral blood, spleen and thymus regardless of the route of ethanol administration (intubation or inhalation in rats, liquid deit for mice). Intubation and liquid diet resulted in a decrease in the ability of lymphocytes from the spleen and peripheral blood to respond to nonspecific mitogens while no such decrease in proliferative ability was noted in rats administered ethanol by inhalation. Adrenalectomy reversed the decrease in ability of lymphocytes to respond to mitogens. The ability to mount a primary immune response was tested using SRBC and TNP-ficol immunization. Animals treated with ethanol showed a decreased ability to respond to the T-cell dependent antigen SRBC but no change in their ability to respond to the T-cell independent antigen TNPO-ficol when compared to controls. The ability to mount a primary immune response to SRBC was not reversed by adrenalectomy. IL2 production was monitored during ethanol administration and was found to be increased in comparison to control at the same time that proliferation was decreased. IL2 receptor numbers also appear to be increased in ethanol treated animals. This project is being incorporated into Z01 AA 00404-01 LPPS.

PHS 6040 (Rev. 1/84)

Project Description

Investigators:

C. Marietta	Physiologist	LPPS, NIAAA
F. Weight	Section Chief	LPPS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
T. Jerrells	Immunologist	Univ of Texas

Objectives:

The objectives of this study are: (1) to determine the effects of alcohol on the immune system in an animal model of alcohol dependent that is controlled and well-defined; (2) to determine the mechanism by which the effects occur; and (3) to compare the effects of alcohol on the immune system using different animals models of alcohol dependence.

Methods Employed:

Male Sprague-Dawley or Lewis rats were made dependent upon ethanol using the intubation technique of Majchrowicz (Psychopharmacologia 43: 245-254, 1975). Rats were sacrificed daily during the period of intubation and their spleens and thymi removed, and peripheral blood samples collected and analyzed. Rats were also studied 1, 3, 5 and 7 days following the termination of ethanol treatment. Thymus glands and spleens were disaggregated and the number of lymphocytes determined. Peripheral blood differentials were determined and lymphocytes isolated for further study. Lymphocytes from the spleen and peripheral blood were cultured in the presence of nonspecific T-cell (Con A and PHA) and B-cell (STM) mitogens and the ability of the lymphocytes to respond to the mitogens was tested in a ^{3}H-thymidine incorporation test. Production of the lymphokine interleukin-2 (IL2) in response to Con A stimulation was also examined. Serum corticosterone levels were determined by RIA. Adrenalectomized and non-adrenalectomized rats were immunized before the start of ethanol treatment as well as at various times during treatment with sheep red blood cells (SRBC) or TNP-ficol. Spleens were obtained at various times after ethanol treatment, disaggregated, counted and and incubated with SRBC or TNP-ficol labeled SRBC and complement in a Cunningham chamber for determination of plaque forming cells (a measure of B-cell activity). Similar experiments were performed on rats exposed to ethanol for 14 days using an inhalation chamber. Similar studies on ethanol's effect on the immune system of C57BL/6 mice were conducted using a liquid diet model of ethanol dependence. Bone marrow cells from ethanol treated rats were cultured in methylcellulose for 2 or 7 days in the presence of saturating concentrations of either erythropoietin or colony-stimulating factor. Colonies were scored as CFU-E or CFU-GM under appropriate conditions. Assays of IL2 binding to Con A stimulated lymphocytes were performed to determine the effect of ethanol on the IL2 receptor. Preliminary studies using fluorescence activated cell sorting techniques were performed to examine changes in lymphocyte subpopulation with ethanol administration.

Major Findings:

Spleen, thymus and peripheral blood lymphocyte numbers decreased after treatment with ethanol in all models studied (intubation or inhalation of rats and liquid diet in mice). Spleen and peripheral blood lymphocytes from intubated rats and mice showed a decreased ability to respond to Con A, PHA, or

STM while rats exposed to ethanol by inhalation did not show a decrease in ability to respond to nonspecific mitogens. Cell counts and proliferative ability returned toward control levels within 7 days after cessation or ethanol treatment in the intubated rats.

The ability of the rats to respond to an immunization with SRBC (a T-cell dependent antigen) also decreased during ethanol administration in intubated rats and mice fed ethanol-containing liquid diet, but the ability of the rats and mice to respond to a T-cell indepedent antigen (TNP-ficol) was unaffected by ethanol treatment.

Corticosterone levls were measured because of indications in the literature that increased corticosteroid levels could cause a decrease in lymphocytes, especially in the thymus. Corticosterone levels were measured in the intubated rats and in mice fed a liquid diet containing ethanol. Corticosterone levels varied throughout the experiment with intubated rats in both the ethanol-treated and control rats. Both ethanol-treated and control rats showed a peak of corticostereone 2 days after beginning intubation, although the ethanol-treated group was significantly elevated ($p < 0.01$). The ethanol-treated rats showed another peak of corticosterone during withdrawal which was not seen in the control group. Elevated corticosterone levels have been demonstrated in the mice by previous investigators (Tabakoff et al., J. Pharm. Pharmac. 30 371-374, 1978).

Adrenalectomy reversed some of the effects of ethanol on the immune system in intubated rats. Defects in lymphocyte proliferation to mitogenic stimuli (Con A, PHA, STM) were reversed by adrenalectomy as was the ethanol induced increase in production of corticosterone. The loss of lymphocytes from the spleen and thymus accompanying ethanol administration was only partially reversed by adrenalectomy. No effects of adrenalectomy on the inability of rats to mount a primary immune response tyo SRBC was noted. Experiments using adrenalectomized mice indicated similar responses.

Examination of the bone marrow from intubated rats or rats exposed to ethanol by inhalation revealed decreased cellularity and a significant lowering of colony growth in the ethanol treated rats compared to the control rats. The red cell progenitors (CFU-E) were preferentially affected, while the myeloid progenitors (CFU-GM) appeared to be relatively resistant to the effects of ethanol.

IL2 (interleukin 2) is necessary for lymphocytes to respond to a mitogen by proliferation. The administration of ethanol to rats by intubation results in an increase in IL2 production when compared to control at the same time that the ability of lymphocytes to respond to mitogens is at the lowest. Data with respect to the IL2 receptors indicate an increase in number of receptors on lymphocytes from ethanol treated rats. More experiments are needed to determine whether the observed changes are due to changes in receptor affinity or some other mechanism (such as uncoupling of a second messenger system) with ethanol treatment.

Preliminary results of FACS studies using monoclonal antibodies to T-cell subsets of both rats and mice indicate that the T-helper subset appears to be the first subset to be affected by ethanol treatment. T-suppressors and B-cells appear to be less ssensitive to the effects of ethanol.

Significance to Biomedical Research and the Program of the Institute:

A significant observation of this study has been that the numbers of lymphocytes in the peripheral blood, spleen and thymus decrease regardless of the method of ethanol administration or species examined. The function of the remaining lymphocytes is imparied in ethanol treated animals where the corticosterone level is high, indicating that ethanol's effect on the immune system may be mediated in part by cortocosteroids. Because lymphocytes are involved in protecting the body from various infectious diseases, these results provide a basis for investigating the increased risk of alcoholics to infectious diseases.

Proposed Course:

Papers are being written and submitted detailing the effects of ethanol administration on the immune system. This project is being incorporated into project #Z01 AA 00404-01 LPPS and will be looking at the ethanol on the immune system using biochemical techniques directed toward signalling proteins in lymphoid tissues.

Publications:

Jerrells TR, Marietta CA, Bone G, Weight FF, Eckardt MJ. Ethanol-associated immunosuppression. Adv in Biochem Psychopharmacol, Ed. Bridge, 44: 1988.

Marietta CA, Jerrells TR, Meagher RC, Karanian JW, Weight FF, Eckardt MJ. Effects of long-term ethanol inhalation on the immune and hematopoietic systems of the rat. Alcoholism: Clin Exp Res 12: 211-214, 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00478-05 LPPS

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.)

Ethanol and Drugs of Dependence: Localizing Effects on Brain Metabolism

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

P.I.:	C. Marietta	Physiologist	LPPS, NIAAA
Others:	M. Eckardt	Section Chief	LCS, NIAAA
	F. Weight	Section Chief	LPPS, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
	K. Grant	Staff Fellow	USP, NIAAA

COOPERATING UNITS (if any)

Physiology Dept., AFRRI, Bethesda, MD (K. Zbicz); Dept. Med., Indiana Univ. Med. Ctr., Indianapolis, IN (T.-K. Li) (G. Szabo), Asst. Prof. Univ. Med. Sch. Szeged, Hungary; (E. London) ARC, NIDA, Baltimore, MD

LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

SECTION

Section on Immunology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS: 0.5	PROFESSIONAL: 0.1	OTHER: 0.4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Previous reports have suggested that localized alterations in brain metabolism occur after administration of ethanol as well as other drugs. General and localized changes in metabolic activity within the brains of rats exposed to various drugs capable of producing physical dependence were studied using the 2-deoxyglucose technique. Specifically, we have studied the chronic administration and subsequent withdrawal as well as the acute effects of ethanol, phenobarbital, diazepam and various peptides on brain glucose metabolism. Similarities and differences were noted in the autoradiographs from rats undergoing withdrawal from ethanol, phenobarbital and diazepam. Among the similarities were the appearance of columns in the frontal sensorimotor cortex and ovoid areas in the cerebellar vermis. The administration of 5 mg/kg of diazepam to an ethanol withdrawing rat resulted in supression of behavioral withdrawal signs and a loss of columnar and ovoid areas in resulting 2-DC autoradiographs. A general decrease in glucose metabolism was noted in Sprague-Dawley rats given ethanol acutely with an increase in metabolism seen in only the superior olive and dentate region of the hippocampus at a blood ethanol concentration of 14 mM. Differences in glucose metabolism were noted in rats that were bred to prefer or not prefer ethanol. Further analysis is being performed to clarify a possible genetic basis for differences seen in ethanol preferring and ethanol non-preferring rats. Experiments are progressing to investigate the effect of various peptides especially peptide T and related peptides) on brain metabolism, using peptide sequences common to the AIDS virus envelope proteins and VIP.

PHS 6040 (Rev. 1/84)

Project Description:

Investigators:

C. Marietta	Physiologist	LPPS, NIAAA
M. Eckardt	Section Chief	LCS NIAAA
F. Weight	Section Chief	LPPS, NIAAA
K. Zbic	Computer Specialist	DCE, AFRRI
T.-K. Li	Professor	Dept. Med. Ind. Univ. Med. Ctr.
G. Szabo	Asst. Professor	Univ. Med School Szeged, Hungary
B. Tabakoff	Scientific Director	NIAAA
K. Grant	Staff Fellow	USP, NIAAA
E. London	Staff Fellow	ARC, NIDA

Objectives:

Various objectives of this project were: (1) to develop dosing schedules that are successful in inducing physical dependence in laboratory rats to ethanol, phenobarbirtal and diazepam; (2) to determine the effects of withdrawal from ethanol, phenobarbital and diazepam on brain metabolism; (3) to determine the effects of acute doses of ethanol, phenobarbital and diazepam on brain metabolism; (4) to determine what effect a given dose to diazepam has on the brain metabolism of rats undergoing ethanol withdrawal; (5) to determine if a given dose of ethanol will cause a different pattern of local cerebral glucose utilization in rats bred to either prefer or not prefer alcoh9ol; and (6) to determine the effect of various peptides related to AIDS virus envelope protein on brain metabolism.

Methods Employed:

Sprague-Dawley rats were rendered dependent upon ethanol, phenobarbital or diazepam by oral intubation of the drug in an appropriate vehicle. Control animals were intubated with the vehicle alone. The time course and dosage schedule varied with the drug under study. Drug levels in the blood were monitored throughout each experiment. When withdrawal symptoms were most intense 2-deoxyglucose (2-DG) was used to determine general and localized changes in brain metabolism. Acute effects were determined by the administration of the drug under investigation i.p. 10 to 30 min before the i.v. injection of 2-DG. For the studies of the peptides related to AIDS virus envelope protein, intracerebroventricular cannulae were implanted several days prior to the experiment and peptides injected i.c.v. 10 min prior to injection of 2-DG.

Major Findings:

We have found statistically significant general and localized increases in glucose metabolism as measured by 2-[^{14}C]-deoxyglucose (2-DG) in rats undergoing withdrawal from ethanol and phenobarbital. Rats undergoing withdrawal from diazepam show statistically significant increases and decreases in glucose utilization. Among similarities noted in withdrawing rats was the appearance of 400 um wide columns in the sensorimotor corte, ovoid areas in the cerebellar vermis and increases in several nuclei associated with the motor system. This is consistent with the preponderance of withdrawal signs that reflect motor functioning, such as hyperactivity, tremors, and spontaneous

convulsions. Differences among the withdrawals included differential increases in the lateral geniculate in diazepam and phenobarbital withdrawals but not ethanol withdrawal.

Acute administration of ethanol produced decreases in glucose metabolism in 20% of the regions examined. Ethanol doses of 0.8, 1.6 and 3.1 g/kg were examined. These doses gave peak blood ethanol levels of 14, 26 and 66 mM, respectively. Increased metabolism was noted in the entate region of the hippocampus and the superior olivaris nucleus at 14 mM ethanol. Decreased metabolism was most dramatic in the median raphe, vestibular, cerebellar vermis and various structures associated with the auditory system.

Diazepam (5 mg/kg) given to ethanol-withdrawing rats yielded autoradiographs in whic the columns were no longer visible. A general decrease in metabolism throughout the brain was noted when compared to ethanol-withdrawing rats. Metabolic rates were simliar to those noted in control rats. These results correlate with an absence of behavioral withdrawal signs in diazepam-treated ethanol-withdrawing rats, indicating that the effect of diazepam on brain glucose utilization during ethanol withdrawal correlates with its effects on the behavioral manifestation of ethanol withdrawal.

The acute administratio of diazepam (5mg/kg) results in decreased glucose utilization throughout the brain. Further statisticaly analysis is being performed to determine if there are localized areas of decreases as well as the general decrease in glucose utilization in rats treated acutely with diazepam.

The administration of 0.5 g/kg ethanol to outbred and selectively bred ethanol preferring and ethanol non-preferring rats resulted in differences between groups in approximately 47% of the structures examined. Acute ethanol administration was associated with decreased cerebral metabolism in the outbred and ethanol preferring groups and with increased metabolism in the ethanol non-preferring group. Almost no differences were noted between outbred and preferring groups while non-preferring rats had higher cerebral metabolic rates. Analysis of additional rats given 2-DG on the increasing or plateau of the blood alcohol curve is in progress to illustrate a possible genetic basis for the different reactions of ethanol preferring and ethanol non-preferring rats to be given dose of ethanol. Preliminary analysis of autoradiographs from rats injected intracerebroventircularly with the ocetapeptide, peptide T, and an analogue of the peptide indicate that both peptides altered local cerebral glucose utilization in rats given injections of the two peptides. Additional experiments using various doses of other related (VIP) active and inactive peptides are necessary to complete this study.

<u>Significance to Biomedical Research and the Program of the Institute</u>:

These studies indicate that there are similarities and differences in glucose metabolism in the withdrawal syndrome seen with ethanol, phenobarbital and diazepam. The similarities and differences may be significant with respect to the pathophysiology of the withdrawal mechanism(s). An additional significant observation is that the autoradiographic picture of the brain of rats undergoing ethanol withdrawal but given 5 mg/kg of diazepam correlates with changes in behavior of the rats given diazepam, a drug used to treat withdrawal symptoms in humans. This suggests the possible use of the 2-DG technique in ethanol-withdrawing rats to screen new drugs for the treatment of ethanol withdrawal. The results of the acute studies indicate that differences in glucose metabolism can be seen with low or high doses of ethanol. The strain

of animals used also appears to be important, as noted in the preliminary results from studies dealing with ethanol preferring and non-preferring rats given the same dose of ethanol and compared to outbred rats of the parent strain. The effects of various peptides on brain glucose metabolism could lead to an understanding of how normal brain metabolism can be affected by foreign peptides.

Proposed Course:

Continued statistical densitometric analyses will be performed on these projects. Several papers are in various stages of preparation for publication. Further investigations of the effects of icv injections of active and inactive peptides associated with the AIDS envelope protein and other related peptides are expected to be carried out by the Unit for Special Projects, NIAAA.

Publications:

Eckardt MS, Campbell GA, Marietta CA, Majchrowicz E, Weight FF. Acute ethanol administration selectively alters localized cerebral glucose metabolism. Brain Res. 444: 53-58, 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00405-01 LPPS

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.)

Detection and regulation of specific cellular phosphoproteins

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

PI:	T.M. Martensen,	Research Chemist	LPPS, NIAAA
Others:	R.L. Kincaid	Section Chief	LPPS, NIAAA

COOPERATING UNITS (if any) Johns Hopkins Univ. (M.D. Lane); Lab. of Molec. Immunoregulation, NCI, NIH (W. Farrar); Univ. of Madrid (M. Mazon) Lab. of Molecular Neurogenetics, NIMH, ADAMHA (B. Martin)

LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

SECTION

Immunology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Ave., Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Immunodecoration of proteins containing phosphotyrosine residues (Tyr-P) was carried out by incubating electroblots of SDS gels with sheep antibodies that bind Tyr-P. Peptide-antibody complexes are detected with affinity-purified anti-sheep IgG conjugated to horse-radish peroxidase. When the procedure was tested with proteins having only phosphorylated serine or threonine sites, no appreciable reaction was observed, indicating the specificity of the method for Tyr-P sites. This immunodetection procedure identified several autophosphorylated growth factor receptor tyrosine kinases (epidermal growth factor (egf) and insulin receptors) on electroblots of tissue extracts as well as phosphotyrosyl-proteins that were modified after cells were exposed to interleukin (IL)-3. In addition, immunoadsorption of Tyr-P-containing proteins was accomplished using anti-phosphotyrosine antibodies immobilized to Protein A-Sepharose or linked covalently to CNBr-activated Sepharose. This approach allowed semi-quantitative adsorption of phosphorylated egf-and insulin- receptors from solubilized tissue preparations.

Studies have been initiated to prepare phosphopeptides comprising phosphorylation sites of several important cellular proteins (e.g. receptors and cytoskeletal proteins) thought to be phosphorylated during cell activation. Novel procedures have been developed for the chemical phosphorylation of serine and threonine residues in these peptides. Specific antibodies against these sites may prove useful in characterizing the physiologic response of cells, especially in lymphocytes and neural tissue, to immuno- or neuro-modulatory influences.

PHS 6040 (Rev. 1/84)

Project Description

Objectives: The role of protein phosphorylation in eukaryotic cellular function is of considerable interest since these modifications may regulate signal transduction events. The primary goals of this project are 1) to develope immunological methods for detection of specific phosphorylated sequences in plasma membrane receptors and other cellular components important to neural and/or immune cell function and 2) to investigate the regulatory mechanisms which affect the interconversion of phospho- and dephospho- forms of these proteins.

Methods Employed:

Tyr-P crosslinked to bovine serum albumin was utilized to prepare anti-TyrP antibodies from sheep. Anti-Tyr-P antibodies were affinity purified from sheep plasma by elution from immobilized Tyr-P using 10 mM Tyr-P. The antibodies were characterized as reported earlier and coupled ("immobilized") to CNBr-Sepharose.

In vivo labeling of cellular protein with $^{32}P_i$ and isolation of plasma membranes was carried out using published methods. Membrane proteins were solubilized in 0.5 % Triton X-100 and incubated at 4°C with immobilized antibodies for about 4 h. After sedimentation of the immune complexes, desorption was achieved by the addition of 50 mM Tyr-P. SDS-PAGE gels were electroblotted in Tris-glycine transfer buffer containing 20% methanol at constant power (20 watts) for 1-2 hr. Nitrocellulose blots were probed with anti-Tyr-P antibodies followed by ^{125}-I-labeled protein-G or affinity purified anti-sheep antibodies (rabbit) conjugated to horse-radish peroxidase.

Major Findings:

Insulin and IGF-1 rapidly stimulated the phosphorylation on tyrosine of a 160 kDa cytosolic protein (pp160) in intact 3T3-Ll adipocytes. Half-maximal pp160 phosphorylation was attained with either 3.5 nM insulin, 18.5 nM IGF-1, or 15 nM IGF-2, in rough agreement with their known *in vitro* potency. Approximately 3 x 10^5 molecules of phosphate were incorporated into pp160 and 0.6 x 105 molecules into the insulin receptor β-subunit per cell. Removal of insulin from stimulated cells resulted in a decrease of phosphate in tyrosyl residues in both pp160 and the β-subunit within 10-15 min. While pp160 remained maximally phosphorylated for up to 60 min in the presence of 100 nM insulin, IGF-1 and IGF-2 at the same concentration induced a transient response that was maximally 50% of that observed with insulin. pp160 was not phosphorylated on tyrosine in response to platelet-derived growth factor (PDGF) or epidermal growth factor (EGF). By virtue of its

cytosolic localization and inability to bind lectins, pp160 does not appear to be a typical glycoprotein growth factor receptor. These data suggest that pp160 is a genuine cellular substrate of the insulin receptor tyrosine kinase, however, the role of pp160 as an intermediary in insulin action remains to be established.

The epidermal growth factor (EGF) receptor in human A431 epidermoid carcinoma cells undergoes a slow ($t_{1/2}$ = 30-40 min) post-translational modification during which it acquires EGF binding capacity. This activation occurs in the endoplasmic reticulum and requires core N-linked glycosylation. By employing both anti-EGF receptor and anti-phosphotyrosine we demonstrated that the EGF receptor also acquired its tyrosine kinase autophosphorylation activity post-translationally ($t_{1/2}$ = 10-15 min). However, unlike the acquisition of EGF binding activity, the appearence of tyrosine kinase activity did not require N-linked glycosylation. Furthermore since the increased tyrosine kinase activity preceded (and, therefore, was independent of) the acquisition of EGF binding capacity, it appears that these two phenomenona reflect separate events.

Interleukin 3 stimulates the proliferation of FCD-P1, a murine myeloid cell line, however the biochemical events subsequent to binding of IL3 have not been investigated in detail. We used an anti-phosphotyrosine antibody to purify proteins containing phosphotyrosine following IL3 administration to FDC-P1 cells. We found phosphorylation of two proteins of 50 (pp50) and 70 (pp70) kDa; in addition to phosphotyrosine both proteins also contained phosphoserine. Together with previous evidence, these results suggest that coactivation of serine/threonine and tyrosine kinase activities is an important element in IL3 signal transduction.

In vivo labeled yeast ^{32}P-phosphoproteins were analyzed by means of an anti-phosphotyrosine antibody, detecting a phosphopeptide of molecular weight 53000 (p53) in Western blots of plasma membrane proteins. Detergent solubilized p53 was adsorbed to antiphosphotyrosine antibody coupled to Sepharose; this preparation was found to have phosphotransferase activity toward casein. Phosphoamino acid analysis after acid hydrolysis showed ^{32}P-phosphoserine and ^{32}P-phosphothreonine indicating that the casein kinase recognized by the antibody was a serine/threonine type protein kinase. A partially purified casein kinase from yeast plasma membrane (M_r = 53,000) was found to be a substrate for protein tyrosine kinases from both rat liver and yeast plasma membrane; the site of modification was confirmed to be a tyrosine residue. These data suggest that the 53 kDa yeast phosphoprotein is a "casein" -type kinase which can be covalently modified by endogenous tyrosine phosphorylation.

Significance to Biomedical Research and the Program of the Institute:

The control of cell growth and function has been related to the phosphorylation of specific sequences within important cytosolic and membrane-bound proteins. Investigating the relationship of these peptide substrates and their kinases will be useful in understanding the molecular basis of normal and pharmacologically-modified (i.e., drug and alcohol dependent) cellular responses.

Proposed Course:

Affinity-purified antibodies will be prepared that are specific for phosphorylated amino acid sequences found in plasma membrane receptors (and other components) known to be regulated by phosphorylation. These antibodies will be used to assess the status of phosphoproteins after effector and/or pharmacologic modulation of cells and tissues. Those agents that prevent or enhance the phosphorylation of specific sequences are likely to markedly alter the normal cellular responses.

Publications:

Madhoff DH, Martensen TM, Lane MD. Tyrosine phosphorylation of a 160 kDa cytosolic protein in 3T3-L1 adipocytes in response to insulin and insulin-like growth factor-1 (IGF-1). Biochem J 1988; 252: 7-15.

Slieker LJ, Martensen TM, Lane MD. Biosynthesis of the epidermal growth factor receptor: post-translational glycosylation-independent acquistion of tyrosine kinase autophosphorylation activity. BBRC 1988; 153; 96-103.

Ferris DK, Martensen TM, Farrar WL. Interleukin-3 stimulation of tyrosine kinase activity in FDC-P1 cells. BBRC, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA00700-04 LPPS

PERIOD COVERED

October 1, 1987-September 30, 1988

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

Ethanol Effects on Membrane-Bound Enzymes

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

PI:	P. Hoffman	Section Chief	LPPS, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
Others:	G. Szabó	Visiting Associate	LPPS, NIAAA
	F. Moses -	Guest Researcher	USP, NIAAA

COOPERATING UNITS *(if any)* University of Illinois, Chicago (J.M. Lee); Westside VA, Chicago (F. De Leon-Jones); Sapporo Medical College (T. Saito); IRP, NINCDS, (G. Eisenhofer); Karolinska Institute, Sweden (S. Borg)

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	1.2	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.)*

It has been hypothesized that the actions of ethanol result from its ability to perturb the structure of neuronal membrane lipids. Changes in the activities of membrane-bound enzymes, which are modulated by the properties of surrounding lipids, may indicate specific sites of action of ethanol within the neuronal membrane, and may persist beyond the time that ethanol is present in tissues, therefore serving as objective measures of alcohol consumption. It has also been suggested that certain enzymes, in particular monoamine oxidase (MAO), may be markers of a genetic predisposition to alcoholism. We previously found that ethanol in vitro inhibits the B-form of MAO in brain and platelet tissue, and that receptor-coupled adenylate cyclase (AC) activity in brain is altered by chronic ethanol ingestion. These enzyme activities were evaluated in platelets of alcoholics and controls. There was no significant difference in MAO activity per se between the two groups, but inhibition of MAO activity by ethanol in vitro was significantly greater, while stimulation of AC activity by fluoride and other agents was significantly lower, in platelets of alcoholics than controls. This latter finding has been replicated in three independent groups of alcoholics and controls. The differences in enzyme properties were long-lasting, and could be used in discriminant analysis to correctly classify 73% of controls and 75% of alcoholics. More work is needed to determine whether the differences are a consequence of alcohol consumption or may reflect a predisposition to alcoholism. Studies of the effects of ethanol in vivo on enzymes of neurotransmitter metabolism made use of an irreversible inhibitor of DOPA decarboxylase, monofluoromethyl DOPA. During ethanol withdrawal, dopamine turnover was reduced in most brain areas, while norepinephrine turnover was increased, particularly in brainstem. These studies further elucidate ethanol effects on neuronal activity, and suggest pharmacological therapies that may be developed to counteract specific symptoms of ethanol withdrawal.

PROJECT DESCRIPTION

Investigators:

P. Hoffman	Section Chief	LPPS, NIAAA
B. Tabakoff	Scientific Director	NIAAA
G. Szabó	Visiting Associate	LPPS, NIAAA
F. Moses	Guest Researcher	USP, NIAAA
G. Eisenhofer	Visiting Fellow	IRP, NINCDS
F. De Leon-Jones	ACOS for Research	Westside VA Med. Ctr., Chicago
J. Lee	Research Associate	Univ. of IL, Chicago
T. Saito	Assistant Professor	Med. College of Sapporo
S. Borg	Professor	Karolinska Inst., Sweden

Objectives:

The goal of this project is to characterize chronic effects of ethanol on the activity of enzymes involved in neurotransmission. Many of these enzymes are membrane-bound, and their activity is modified by the properties of the lipids that surround them. As a result, the enzyme activities can serve as specific probes to elucidate the site of action of ethanol within the neuronal membrane. Changes in enzyme activity following chronic, in vivo, ethanol ingestion may reflect alterations in the properties of selected areas of neuronal or other cell membranes. Such changes may be associated with neuroadaptive responses (tolerance, physical dependence) to ethanol, or pathological effects of ethanol and, if they persist beyond the time that ethanol is present in the body, might serve as diagnostic tools reflecting chronic ethanol consumption. Other diagnostic tools that would be of value to clinicians include biochemical markers that might identify individuals at risk for developing alcoholism. Previous work with monoamine oxidase (MAO; Tabakoff et al., Psychopharmacol. 87: 152, 1985) and adenylate cyclase (Saito et al., J. Neurochem. 44: 1037, 1985) suggested that these enzyme activities might be useful as such diagnostic tools. An objective of this project is to evaluate these enzyme activities in platelets of alcoholic and control subjects.

To elucidate specific sites and mechanisms of action of ethanol in the CNS, effect of ethanol in vivo on neurotransmitter metabolism--which reflects neuronal activity---has been evaluated.

Methods:

Ethanol Treatment. For chronic ethanol treatment, male C57BL/6NCR mice (23-25 g) were individually housed and fed a liquid diet containing ethanol or an equicaloric amount of sucrose (controls) for seven days (Ritzmann and Tabakoff, J. Pharmacol. Exp. Ther. 199: 158, 1976). The amount of diet offered to the control animals was adjusted daily to match the amount consumed by the ethanol-fed mice. This treatment regimen produced functional tolerance to and physical dependence on ethanol.

Brain Catecholamine Turnover. Male C57BL/6NCR mice were fed ethanol in a liquid diet for seven days, as described above. At the time of withdrawal, they were injected ip with DL-alpha-monofluoromethyldopa, and were sacrificed at various times after injection. Brains were removed and cortex, hippocampus, striatum, brainstem and cerebellum were dissected. Brain amine and metabolite levels were analyzed by HPLC to determine rates of catecholamine turnover (Eisenhofer et al., Clin. Chem. 32: 2030, 1986).

MAO Activity. MAO activity and inhibition of activity by ethanol were measured in human platelets. Platelets were prepared from whole blood which was obtained by venipuncture from alcoholic and control individuals who had given informed consent. The platelets were lysed in hypotonic buffer, frozen and thawed to provide membranes for enzyme assays. MAO activity was assayed by a modification of the spectrophotometric method of Tabakoff and Alivisatos (Anal. Chem. 44: 427, 1972), using dimethylaminobenzylamine (DAB) as substrate, and also by a modification of a radioisotopic assay using ^{14}C-phenylethylamine (PEA) as substrate (Wurtman and Axelrod, Biochem. Pharmacol. 12: 1439, 1963).

Adenylate Cyclase Activity. Platelets were obtained from whole blood, and platelet membranes (30,000xg pellet) were prepared. Adenylate cyclase activity was assayed by determining the conversion of ^{32}P-ATP to ^{32}P-cAMP by previously described methods (Saito et al., J. Neurochem. 44: 1037, 1985).

Major Findings:

Two markers that would be of use to the clinician are 1) biochemical measures that would accurately reflect recent long-term consumption of alcohol and 2) biochemical markers associated with a predisposition to alcoholism. Platelet MAO activity has often, but not always, been found to be lower in alcoholics than controls. This enzyme activity has been suggested as a genetically-influenced marker for alcoholism. Another characteristic of platelet MAO activity that might distinguish alcoholic and non-alcoholic subjects is inhibition of MAO by ethanol in vitro (Tabakoff et al., Psychopharmacol. 87: 152, 1985). In animal studies, we have found that chronic ingestion of ethanol results in characteristic changes in receptor-coupled adenylate cyclase (AC) in brain (Saito et al., J. Neurochem 48: 1817, 1987; Valverius et al., Mol. Pharmacol. 32: 217, 1987). These changes reversed within a few days after ethanol withdrawal, and might therefore provide a reliable index of

recent alcohol consumption. To evaluate these enzyme activities we used a population of alcoholics and age-matched controls. Subjects with medical, neurologic or major psychiatric disorders were excluded. Our results showed no significant difference between alcoholics and controls in platelet MAO activity per se (using phenylethylamine as substrate). However, inhibition of platelet MAO activity by ethanol was significantly greater in alcoholics than in controls, when high substrate concentrations were used. There were also differences in platelet AC activity between the two groups. Although basal activity was not significantly different, stimulation of AC activity by fluoride, guanine nucleotides or prostaglandin E_1 was lower in platelets of alcoholics than controls. Statistical analysis showed that the differences observed in both enzyme activities were not associated with race, smoking behavior or illicit drug use, and, interestingly, there were no significant correlations of the enzyme activities with age, duration of problems with alcohol or time since the last alcoholic drink. Discriminant analysis, using the values for inhibition of MAO activity by ethanol and for fluoride-stimulated AC activity, correctly classified 75% of alcoholics and 73% of controls.

We have recently completed preliminary studies of platelet enzyme activities in another group of male alcoholics and controls from St. Göran's Hospital, Stockholm, Sweden. As in the earlier study, there was no significant difference in basal AC activity between controls and alcoholics, while fluoride- and Gpp(NH)p-stimulated AC activities were significantly lower in platelets of alcoholics than controls. Similarly, in platelets from groups of alcoholic and control subjects at the VA Medical Center in Charleston, South Carolina (provided by Dr. Ray Anton), basal AC activities were similar while fluoride-stimulated AC activity was lower in platelets of alcoholics than controls. The differences in platelet AC activity between alcoholics and controls in our investigations seemed similar to the changes seen in animal studies, in that basal activity was not affected, while stimulation by a number of agents acting via G_s was decreased. It is possible that in platelets of alcoholics, as proposed for brain membranes of ethanol-fed mice, the amount or function of G_s may be altered.

When the data from the Swedish alcoholics were analyzed statistically, a significant negative correlation was found between fluoride-stimulated AC activity and time since the last alcoholic drink, both in controls and in alcoholics. Although this strong correlation was not observed in our earlier study, it was found in our earlier work that platelet AC activity remained lower in alcoholics, compared to controls, after reported abstention from alcohol for one to four years. Similarly, inhibition of MAO activity by ethanol did not correlate significantly with time since the last alcoholic drink. These findings support the possibility that the platelet enzyme characteristics could represent a genetically-influenced, inherent characteristic associated with a predisposition to alcoholism. However, more work and different approaches are needed in order to determine whether the differences in platelet enzyme characteristics are inherent or represent a consequence of long-term chronic ethanol ingestion.

Studies of catecholamine turnover in mouse brain are important for evaluating the sites of action of ethanol in the CNS, and for determining whether the effects of ethanol on neurotransmitter receptor characteristics are direct or indirect. The use of monofluoromethyl DOPA, an irreversible inhibitor of DOPA decarboxylase, allows measurement both of the rate of L-DOPA accumulation, and the rates of norepinephrine and dopamine depletion (Palfreyman et al., J. Pharmacol. Meth. 11: 239, 1984). These data allow a more accurate assessment of catecholamine turnover rates than is possible with many other methods that have been used. Our studies, in C57BL mice, showed little change in brain dopamine or norepinephrine turnover during chronic ethanol ingestion. However, at the time of peak withdrawal symptomatology (i.e., 8 hr after withdrawal), dopamine turnover in striatum and other brain areas was markedly reduced, while norepinephrine turnover, particularly in brainstem, was increased. The findings indicate that, during ethanol withdrawal, there are brain region-specific changes in neurotransmitter turnover--presumably reflecting neuronal activity--that may reflect, or contribute to, particular signs and symptoms of ethanol withdrawal. The reduced dopamine turnover may be especially important in contributing to the incidence of extrapyramidal symptoms during withdrawal, in view of earlier findings of dopamine receptor subsensitivity at this time.

Our data indicate that changes in brain β-adrenergic receptor characteristics that were previously observed in ethanol-fed mice at the time of ethanol withdrawal do not result from altered neurotransmitter turnover, since the changes in receptor characteristics occurred prior to the changes in turnover. The changes in catecholamine turnover that occur during ethanol withdrawal suggest that certain pharmacological agents may be of use in counteracting specific aspects of the ethanol withdrawal syndrome.

Significance to Biomedical Research and the Program of the Institute:

The finding that characteristics of platelet enzymes differ in alcoholics and controls has the potential to provide a simple and reliable diagnostic tool to identify individuals who have consumed ethanol chronically, or who have a genetic predisposition to problems with alcohol. Such objective, easily-assayable measures would be of value to clinicians dealing with alcoholic patients. These studies also provide a model system in which to investigate the mechanism by which ethanol may produce alterations in the activity of particular membrane-bound enzymes.

The studies of the effects of chronic ethanol ingestion on catecholamine turnover have made use of a more precise method for neurochemical evaluation of neuronal activity, and the have more clearly defined the effects of ethanol on that activity. These investigations may suggest pharmacological treatments that could be of use in combatting certain symptoms of ethanol withdrawal.

Proposed Course:

The question of whether differences in platelet enzyme activities represent a consequence of ethanol ingestion, or may be an inherent characteristic, will be addressed initially by comparing enzyme characteristics in platelets and lymphocytes of individuals who are "family history-positive" and "family history-negative" for alcoholism. Studies of the content of platelet and lymphocyte G_s in controls and alcoholics, by Western blot analysis, will be performed, and a radioimmunoassay or ELISA to more quantitatively measure G_s in these and other tissues will be developed. The studies on catecholamine turnover have been completed.

Publications:

Tabakoff B, Hoffman PL, Lee JM, Saito T, Willard B, DeLeon-Jones F. Differences in platelet enzyme activity between alcoholics and nonalcoholics, N Engl J Med 1988;318:134-9.

Hoffman PL, Lee JM, Saito T, Willard B, DeLeon-Jones F, Valverius P, Borg S, Tabakoff B. Platelet enzyme activities in alcoholics, Proc K Eriksson Memorial Symposium: Alcohol and Genetics, Sapporo, Japan, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA00702-04 LPPS

PERIOD COVERED

October 1, 1987 - September 30, 1988

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

Ethanol Modification of Neurotransmitter Receptor-Effector Coupling

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

PI:	P. Hoffman	Section Chief	LPPS, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
Other:	P. Valverius	Visiting Fellow	LPPS, NIAAA
	P. T. Nhamburo	Visiting Fellow	LPPS, NIAAA
	A. R. Rius	Visiting Fellow	LPPS, NIAAA
	F. Moses	Guest Researcher	USP, NIAAA

COOPERATING UNITS (*if any*)

Metabolic Disease Branch, NIDDKD (A. M. Spiegel)

LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

SECTION

Section on Receptor Mechanisms

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.7	2.7	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Ethanol selectively alters the function of neurotransmitter and neuromodulator receptors in the CNS. These changes may contribute to the acute, intoxicating effects of ethanol, and adaptations in receptor function may be associated with ethanol tolerance and/or physical dependence. Previous work showed decreased stimulation of cerebral cortical adenylate cyclase (AC) activity by beta-adrenergic agonists and guanine nucleotides, and decreased high-affinity beta-adrenergic agonist binding, in cerebral cortex of mice fed ethanol chronically. The data suggested an alteration in the properties of Gs, the stimulatory guanine nucleotide binding protein, in these mice. This hypothesis was supported by a demonstration of reduced stimulation of cerebral cortical AC activity by other agents that act through Gs in ethanol-fed mice, compared to controls, and reduced availability for cholera toxin-catalyzed ADP-ribosylation of a protein migrating like Gs on SDS-polyacrylamide gels. In contrast, there was no difference between control and ethanol-fed mice in pertussis toxin-catalyzed ADP-ribosylation of cerebral cortical proteins or, in initial studies, in the quantity of Go-alpha or Gi-alpha measured by Western blot analysis. Chronic ethanol ingestion thus appears to selectively affect the amount and/or function of one of a family of similar G proteins. The effects of ethanol on receptor-AC coupling are brain region-specific. Changes in beta-adrenergic agonist-stimulated AC activity and high-affinity agonist binding were similar in hippocampus and cortex of ethanol-fed mice, compared to controls, while little change was observed in cerebellum. This regional specificity was supported by autoradiographic analysis of high-affinity forskolin binding, which is believed to reflect binding to the Gs-alpha-AC complex. The regional specificity of the effects of ethanol on receptor-effector coupling may contribute to specific behavioral responses to chronic ethanol ingestion.

PROJECT DESCRIPTION

Investigators:

P. Hoffman	Section Chief	LPPS, NIAAA
B. Tabakoff	Scientific Director	NIAAA
P. Valverius	Visiting Fellow	LPPS, NIAAA
P. T. Nhamburo	Visiting Fellow	LPPS, NIAAA
A. R. Rius	Visiting Fellow	LPPS, NIAAA
F. Moses	Guest Researcher	USP, NIAAA
A. M. Spiegel	Chief	MD, NIDDKD

Objectives:

The goal of this project is to determine the effects of ethanol on neurotransmitter receptor-effector coupling processes in brain. It has been postulated that the acute effects of ethanol (e.g., intoxication), as well as adaptation to these effects (tolerance, physical dependence) are mediated by changes in neuronal membrane lipid properties. However, the functional moieties within the membranes are proteins, such as receptors. Our previous work showed that ethanol has specific sites of action within the receptor-adenylate cyclase (AC) systems in brain, selectively affecting the protein-protein interactions that mediate receptor-effector coupling processes. Our objective in this project continues to be a determination of the specificity and mechanism of action of ethanol on receptor-coupled AC systems, and other receptor-effector systems, and the role of these systems in neuroadaptation to ethanol. These studies define the sites of action of ethanol within the CNS, and determine if adaptive changes in the receptor-effector systems occur at the initial sites of action of ethanol, and may be associated with specific behavioral aspects of ethanol tolerance or physical dependence.

Methods:

Ethanol Treatment. For chronic ethanol treatment, male C57BL/6NCR mice (23-25 g) were individually housed and fed a liquid diet containing ethanol or an equicaloric amount of sucrose (controls) for seven days (Ritzmann and Tabakoff, J. Pharmacol. Exp. Ther. 199: 158, 1976). The amount of diet offered to the control animals was adjusted daily to match the amount consumed by the ethanol-fed mice. This treatment regimen produced functional tolerance to and physical dependence on ethanol. Animals were sacrificed either at the time of withdrawal or at various times after withdrawal.

Adenylate Cyclase Assay. Animals were killed by decapitation, brains were removed, and a 48,000xg membrane fraction was prepared as previously described (Luthin and Tabakoff, J. Pharmacol. Exp. Ther. 228: 579, 1984). Adenylate cyclase activity was measured by determining the production of ^{32}P-cyclic AMP from ^{32}P-ATP (Tabakoff and Hoffman, J. Pharmacol. Exp. Ther. 208: 216, 1979).

Beta-Adrenergic Receptor Binding Assay. Animals were killed by decapitation, and brains were rapidly removed. Cerebral cortical, hippocampal and cerebellar membranes (48,000xg membrane fraction) were prepared as described above. Antagonist (^{125}I-iodocyanopindolol; ICYP) binding was assayed according to the method of Engel et al. (N-S. Arch. Pharmacol. 317: 277, 1981). Agonist (isoproterenol; ISO) binding was assayed by displacement of bound ICYP. Data were analyzed using the LIGAND program (Munson and Rodbard, Anal. Biochem. 107: 220, 1980).

Forskolin Binding. Brains slices (20 µm) of C57BL/6NCR mice were thaw-mounted on gelatin-coated glass slides and were incubated with 20 nM ^{3}H-forskolin, in the presence or absence of 10 µm Gpp(NH)p, as previously described (Gehlert et al., Brain Res. 361: 351, 1985). Nonspecific binding was determined in the presence of 20 µm unlabeled forskolin. Slides were exposed to X-ray film, and autoradiograms were analyzed by computerized densitometry.

Quantitation of Guanine Nucleotide Binding Proteins. A 48,000xg cerebral cortical membrane preparation of C57BL/6NCR mice was incubated with α-^{32}P-NAD in the presence or absence of activated cholera or pertussis toxin (Ribiero-Neto et al., Meth. Enzymol. 109: 566, 1985). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, Nature, New Biol. 227: 680, 1970), and ^{32}P-ADP-ribose incorporation was determined by densitometry of autoradiograms. For Western blot analysis, proteins in a similar membrane preparation were separated by SDS-PAGE and transferred to nitrocellulose paper by electroblotting. The blots were incubated with polyclonal antibodies to $G_{s\alpha}$, $G_{i\alpha1}$, $G_{i\alpha2}$ or $G_{o\alpha}$ (provided by Dr. A. Spiegel, NIDDKD) and then with ^{125}I-labeled protein A. The blots were exposed to X-ray film and autoradiograms were analyzed as described above or by scanning on a Gilford spectrophotometer. In some instances, protein bands on blots were also visualized with second antibody coupled to diaminobenzidine, the radioactive bands were cut out, and bound labeled protein A was quantitated by liquid scintillation spectrometry.

Major Findings:

The results obtained indicate that chronic ethanol ingestion produces selective and brain region-specific effects on G_s, the stimulatory guanine nucleotide binding protein. These changes may be important for neuroadaptive or pathological responses to ethanol. We have previously shown that chronic ethanol ingestion by mice results in decreased stimulation of cerebral cortical adenylate cyclase (AC) activity by guanine nucleotides and β-adrenergic agonists. Our more recent studies showed that high-affinity agonist binding to the cerebral cortical β-adrenergic receptor was undetectable in ethanol-fed mice, although readily distinguished in control mice. The form of the β-adrenergic receptor with high affinity for agonist is believed to reflect the formation of a complex of the receptor with G_s, the stimulatory guanine nucleotide binding protein. Together, these data suggested that chronic ethanol ingestion resulted in an alteration in the amount or function of cerebral cortical G_s. This postulate was supported by

our finding that stimulation of cerebral cortical AC activity by other agents that act through G_s (e.g., forskolin, vasoactive intestinal peptide (VIP)) was also reduced in cerebral cortical membranes of ethanol-fed mice, compared to controls. We have quantitated cerebral cortical G_s and other G proteins by two methods. In the first, cholera and pertussis toxin-induced labeling with ^{32}P-ADP-ribose was assessed, and, in the second, G proteins were quantitated by Western blot analysis, using specific antibodies raised by Dr. Alan Spiegel, NIDDKD, against synthetic peptides corresponding to sequences of particular G proteins. In cerebral cortical membranes from mice that had ingested ethanol chronically, there was a selective decrease in the amount of cholera toxin-induced labeling of a protein with approximate molecular mass of 46 kDa (i.e., there was no change in cholera toxin-induced labeling of other proteins). The magnitude of the decrease in cholera toxin-induced ADP-ribosylation was comparable to the change in Gpp(NH)p-stimulated AC activity in cerebral cortex of ethanol-fed mice. There was no apparent change in pertussis toxin-induced labeling. G_o, as well as various forms of G_i, are subject to ADP-ribosylation catalyzed by pertussis toxin, and G_o is present in very high quantities in brain, such that changes in labeling of G_i might not be detectable by this method. However, using antibodies that specifically react with $G_{o\alpha}$, $G_{i\alpha 1}$ or $G_{i\alpha 2}$, no change in the quantity of these proteins was apparent in ethanol-fed mice. The data suggest that chronic ethanol ingestion results in a decrease in $G_{s\alpha}$ that has functional consequences in terms of AC stimulation. To further elucidate the mechanism of the observed change in G_s, we have initiated studies of $G_{s\alpha}$ content by Western blot analysis, as well as studies of the synthesis of G_s, G_o and G_i by Northern blot analysis. Preliminary results show a large quantity of mRNA for G_s in mouse cortex, with lower amounts of mRNA for G_o and G_i. This finding confirms earlier work, and has been suggested to indicate rapid turnover of G_s in brain. Studies are underway to compare G protein mRNA in brains of control and ethanol-fed mice.

The change in the properties of G_s induced by chronic ethanol ingestion appears to be specific for certain brain regions. In hippocampus, as in cerebral cortex of ethanol-fed mice, there was decreased stimulation of AC activity by β-adrenergic agonists, compared to controls, and high-affinity agonist binding was undetectable. However, in cerebellum of the same mice, stimulation of AC activity was unchanged, and both high- and low-affinity agonist binding sites were detectable. We have further evaluated the regional specificity of ethanol's effects by quantitative autoradiographic analysis of forskolin binding in mouse brain. High-affinity forskolin binding is believed to reflect an interaction of forskolin with activated G_s-AC complex, and should therefore be an indicator of G_s status. We have also examined the effect of Gpp(NH)p on forskolin binding in brains of control and ethanol-fed mice. We found significantly lower forskolin binding in many brain areas, including cortex, hippocampus, thalamus and hypothalamus of ethanol-fed mice, compared to controls. However, in cerebellum and also in the nucleus accumbens and central nucleus of the thalamus there was no difference in forskolin binding between the two groups. In addition, in most brain areas of ethanol-fed mice Gpp(NH)p did not significantly increase forskolin binding, while Gpp(NH)p enhanced binding in brains of control mice by 50-100%. These

data confirm the regional specificity of the actions of alcohol on receptor-AC coupling in brain, and are consistent with the interpretation of an altered quantity and/or function of G_s in particular brain regions of ethanol-fed mice.

Significance to Biomedical Research and the Program of the Institute:

Ethanol is generally considered to be a drug that does not act at a specific receptor, and a hypothesis to explain the CNS effects of ethanol is based on its ability to perturb neuronal membrane lipid structure in a relatively non-specific manner. In contrast, our data demonstrate that ethanol has specific and selective sites of action on receptor-effector coupling processes, in particular at G_s, the stimulatory guanine nucleotide binding protein, in certain brain areas. Changes in the function of the ubiquitous receptor-coupled AC systems as a result of chronic ingestion of ethanol could produce neurochemical imbalances that may contribute to specific signs of ethanol tolerance and/or physical dependence, and suggest means for modulating these processes. These studies also provide insight into basic mechanisms of receptor-effector coupling, and the physiological adaptive responses of these systems.

Proposed Course:

The quantitative changes in G_s that appear to occur after chronic ethanol ingestion will be confirmed, as will the lack of change in G_0 and G_i. A radioimmunoassay or ELISA for more accurate quantitation of G_s will be developed. Studies of the synthesis of the G proteins in ethanol-treated animals (i.e., quantitation of mRNA) will be performed, both by Northern blot analysis and by in situ hybridization. Additionally, $G_{s\alpha}$, $G_{0\alpha}$ and $G_{i\alpha 1}$ and $G_{i\alpha 2}$ will be localized and quantitated in brains of control and ethanol-fed animals by immunocytochemistry. If appropriate, studies on other possible mechanisms that could contribute to reduced receptor-effector coupling, including changes in the β/γ subunits of the G proteins, and in membrane-bound ribosylation factors, will be initiated.

Publications:

Khatami S, Hoffman PL, Shibuya T, Salafsky B. Selective effects of ethanol on opiate receptor subtypes in brain, Neuropharmacology 1987;26:1503-7.

Valverius P, Hoffman PL, Tabakoff B. Effect of ethanol on mouse cerebral cortical β-adrenergic receptors, Mol Pharmacol 1987;32:217-22.

Tabakoff B, Hoffman PL. Biochemical pharmacology of alcohol. In: Psychopharmacology: The Third Generation of Progress, Raven Press, New York 1987;1521-26.

Kwast M, Tabakoff B, Hoffman PL. Effect of ethanol on cardiac beta adrenergic receptors, Eur J Pharmacol 1987;142:441-5.

Szabó G, Hoffman PL, Tabakoff B. Forskolin promotes the development of ethanol tolerance in 6-hydroxydopamine-treated mice, Life Sci 1988;42:615-21.

Tabakoff B, Hoffman PL, McLaughlin A. Is ethanol a discriminating substance? Semin Liver Dis 1988;8:26-35.

Tabakoff B, Hoffman PL, Liljequist S, Eckardt MJ, Marietta CA, Majchrowicz E, Weight FF. Effects of chronic ethanol ingestion on brain metabolism and receptor systems, Proc Intl Symp Med Biol Prob Alcoholism, in press.

Tabakoff B, Luthin G, Saito T, Hoffman PL. Ethanol's actions on receptor-effector coupling in brain, Proc 5th Intl Conf Rec Adv Biomed Aspects Alcohol and Alcoholism, in press.

Tabakoff B, Hoffman PL. Adaptive responses to ethanol in the central nervous system. In: Alcoholism: Biomedical and Genetic Aspects, Pergamon Press, New York, in press.

Nhamburo PT, Hoffman PL, Tabakoff B. Cholera-toxin-induced ADP-ribosylation of a 46 kDa protein is decreased in brains of ethanol-fed mice, Adv Alc Subst Abuse, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA00703-04 LPPS

PERIOD COVERED
October 1, 1987 - September 30, 1988

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

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

PI:	P. Hoffman	Section Chief	LPPS, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
Other:	L. Liu	Guest Researcher	LPPS, NIAAA
	J. Dave	Visiting Associate	LPPS, NIAAA
	S. Culp	Physical Sci. Tech.	LPPS, NIAAA
	H. Ishizawa	Visiting Fellow	LPPS, NIAAA
	P. Rathna Giri	Visiting Associate	LPPS, NIAAA

COOPERATING UNITS (*if any*)
LCB, NIMH (M. Brownstein, S. Young)

LAB/BRANCH
Laboratory of Physiologic and Pharmacologic Studies

SECTION
Section on Receptor Mechanisms

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	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.*)

Arginine vasopressin (AVP) and related peptides, when administered exogenously, prolong the duration of tolerance to ethanol. Using peptide agonists and antagonists that interact selectively with vasopressin V1 and V2 receptors, we characterized as V1 the receptors in brain that mediate the ability of vasopressin to maintain ethanol tolerance. We have also characterized vasopressin binding sites in brain by quantitative autoradiography. The highest concentration of binding sites was in the lateral septum, with lower densities in dorsal hippocampus, hypothalamus and thalamic areas. Displacement studies with V1- and V2-selective agonists and antagonists indicated that binding sites in the lateral septum have characteristics of V1 receptors. 6-OHDA treatment significantly reduced the number of AVP binding sites in lateral septum, while 5,7-DHT treatment did not. Previous work showed that intact brain noradrenergic systems are necessary for AVP to maintain ethanol tolerance; thus, AVP may act at V1 receptors on terminals of noradrenergic neurons to alter neurotransmitter release and thus influence tolerance. Another possible (postsynaptic) mechanism of action involves stimulation of expression of the proto-oncogene c-fos, which has previously been postulated to be involved in learning or memory. AVP increases c-fos expression in lateral septum after icv injection. This may represent a direct or indirect action of AVP. Our work also showed that endogenous AVP plays a role in maintenance of ethanol tolerance, leading us to investigate AVP synthesis during chronic ethanol treatment. In mice and rats exposed to ethanol by different methods, hypothalamic AVP mRNA was decreased. However, AVP levels in plasma varied, depending on the method of ethanol treatment, suggesting that other factors such as dehydration or stress may influence these levels. Understanding the role and mechanism of action of AVP in development, expression and dissipation of tolerance to ethanol may lead to benign means for the manipulation of tolerance and, possibly, of ethanol intake.

PROJECT DESCRIPTION

Investigators:

P. Hoffman	Section Chief	LPPS, NIAAA
B. Tabakoff	Scientific Director	NIAAA
L. Liu	Guest Researcher	LPPS, NIAAA
J. Dave	Visiting Associate	LPPS, NIAAA
H. Ishizawa	Visiting Fellow	LPPS, NIAAA
S. Culp	Physical Sci. Tech.	LPPS, NIAAA
P. Rathna Giri	Visiting Associate	LPPS, NIAAA
M. Brownstein	Chief	LCB, NIMH
S. Young	Staff Fellow	LCB, NIMH

Objectives:

Arginine vasopressin (AVP), a mammalian antidiuretic hormone, and structurally-related peptides, can maintain functional tolerance to ethanol in mice and rats, once that tolerance has been established. The investigations demonstrating this effect of AVP have involved administration of exogenous hormone or its analogs. The goals of this project include characterization of the CNS receptors and neurochemical mechanisms involved in the action of the hormone, evaluation of the role of endogenous hormone in the maintenance of tolerance, and a study of the effects of acute and chronic ethanol administration on the biosynthesis and release of AVP and oxytocin.

Methods:

Chronic Ethanol Treatment. Male C57BL/6NCR mice were made tolerant to and physically dependent on ethanol by feeding them a liquid diet containing 7% ethanol (Ritzmann and Tabakoff, J. Pharm. Exp. Ther. 199: 158, 1976). Control mice were pair-fed a diet containing an equicaloric amount of sucrose. Rats were exposed to ethanol vapor in an inhalation chamber for 24 hr/day (continuous exposure) for eight days, as described by Karanian et al. (Alc: Clin. Exp. Res. 10: 443, 1986), or for 15 hr/day (intermittent exposure) for 12 days (Tabakoff and Culp, Alc: Clin. Exp. Res. 8: 495, 1984). In the latter case, chamber and blood ethanol levels were measured each day by gas chromatography, and after withdrawal from ethanol, rats were tested for tolerance to ethanol by measuring blood ethanol levels at regain of the aerial righting reflex following a challenge dose of ethanol (Tabakoff and Culp, Alc: Clin. Exp. Res. 8: 495, 1984).

Vasopressin Biosynthesis. To study the effects of ethanol on vasopressin synthesis and release, a radioimmunoassay was used to measure vasopressin levels in rat hypothalamus, posterior pituitary, plasma, anterior pituitary or testes (Dorsa and Bottemiller, Brain Res. 242: 151, 1982). RNA was isolated by previously-described methods, and vasopressin mRNA was quantitated by slot blot and Northern blot techniques, using a synthetic oligodeoxynucleotide

probe or a riboprobe generated from a fragment of the vasopressin gene in the pGEM-3 system. The probes were labeled with ^{32}P (Chirgwin et al., Biochem. 18: 5294, 1979; Maniattis et al., Molecular Cloning, Cold Spring Harbor, 1982). Autoradiograms of slot or Northern blots were quantitated by densitometry on a Gilford spectrophotometer or by computerized image analysis. For in situ hybridization of vasopressin mRNA, 10 µm slices of mouse brain were mounted on glass slides and incubated with the riboprobe that had been labeled with ^{35}S by including labeled thionucleotides in the reaction mixture. Slides were exposed to X-ray film and the autoradiograms were quantitated by computerized densitometry. The slides were then coated with photographic emulsion, the emulsion was developed, and the underlying tissue was stained with thionine.

c-Fos Expression. To measure the effect of AVP on expression of the proto-oncogene c-fos in brain, C57BL mice were implanted with intracerebroventricular (icv) cannulae and were injected with AVP or other peptides in artificial CSF vehicle. Mice were decapitated, and the lateral septal area, hippocampus, cerebral cortex and hypothalamus were dissected. RNA was extracted by previously described methods and c-fos mRNA was quantitated by Northern blot analysis using a cDNA probe (Lofstrand Laboratories, MD) that was labeled with ^{32}P by nick translation. Autoradiograms were quantitated by densitometry as described above.

Vasopressin Receptor Binding. Vasopressin receptors in the CNS were characterized by quantitative autoradiography. Brain slices (10 µm) were thaw-mounted on chrome-alum gelatin-coated glass slides and were incubated with ^{3}H-labeled AVP or ^{3}H-labeled $d(CH_2)_5$ Tyr(ET)VAVP (a V_1-selective antagonist; New England Nuclear Corp., Boston, MA) at room temperature for 30 min. Non-specific binding was assessed with unlabeled AVP. After drying, slides were exposed to X-ray film and autoradiograms were analyzed densitometrically using a computerized image analysis system. To evaluate the effects of 6-OHDA or 5,7-DHT on AVP binding, the neurotoxins were administered icv (6-OHDA, 27 µg free base; 5,7-DHT 11 µg free base). Desmethylimipramine (25 mg/kg) was administered prior to 5,7-DHT.

Major Findings:

We had previously shown that AVP, given peripherally or intracerebroventricularly (icv), can maintain functional ethanol tolerance in mice that have developed such tolerance, even in the absence of further ethanol ingestion. Our more recent studies, using agonists and antagonists selective for V_1 or V_2 vasopressin receptors, demonstrated that V_1 receptors in brain mediate the effect of AVP on maintenance of ethanol tolerance. Autoradiographic studies have been performed to localize and characterize vasopressin binding sites in brain. Using ^{3}H-AVP as a ligand, we have demonstrated specific binding in several mouse brain areas. The highest density of binding sites is in the lateral septum, with other binding sites in dorsal hippocampus, bed nucleus of the stria terminalis, hypothalamus and thalamus. Scatchard analysis of binding in the lateral septum revealed a single binding site for AVP (K_D 5.9

nM; B_{max}, 50 fmol/mg protein). The ^{3}H-labeled V_1 antagonist bound to sites in the lateral septum with a higher affinity than AVP (K_D 0.7 nM; B_{max} 58 fmol/mg protein). Furthermore, ^{3}H-AVP binding could be displaced completely by a V_1-selective agonist or antagonist, while V_2-selective compounds were much less effective. These data suggested that the vasopressin binding site in lateral septum has the characteristics of a V_1 receptor, as has also been suggested by others. Our previous work showed that the maintenance of tolerance by vasopressin depended on the presence of intact noradrenergic systems in brain. Since, in the periphery, V_1 receptors mediate peptide-induced increases in intracellular calcium, it is possible that AVP could interact with neuronally-localized V_1 receptors in the lateral septum to promote the release of norepinephrine and thus modulate tolerance. Treatment of mice with 6-OHDA, at a dose shown previously to partially deplete norepinephrine while having little effect on dopamine, significantly reduced the number of AVP binding sites in the lateral septum. Injection of 5,7-DHT, a neurotoxin that destroys serotonin-containing terminals, did not alter AVP binding. The results indicate that at least some of the AVP binding sites in the lateral septum may be localized on norepinephrine-containing terminals, and thus AVP may act presynaptically to alter neurotransmitter release.

Another possible mechanism of action of AVP, that would involve an action at postsynaptic receptors, is the stimulation of c-fos expression. C-fos is a proto-oncogene whose products are involved in growth and differentiation. Its expression is stimulated by neurotransmitters, as well as growth factors, and it has been postulated that fos proteins may be important in learning or memory (Greenberg et al., Science 234: 80, 1986). Tolerance is a neuroadaptive process that has been likened to learning, and since c-fos expression can be enhanced by increases in intracellular calcium, it seemed possible that the effect of AVP on tolerance, as well as on memory-related processes, might involve an increase in c-fos expression. When mice were injected icv with AVP, an increase in c-fos mRNA was seen in the lateral septum, with a lesser increase in hippocampus. A V_1-selective agonist also increased c-fos expression, while a V_2-selective agonist was less effective. Thus, vasopressin may influence neuroadaptive processes by stimulating the expression of this proto-oncogene in the CNS, either directly or secondary to increasing neurotransmitter release.

Most of our studies of the effect of AVP on ethanol tolerance have involved exogenous administration of the peptide. We are also interested, however, in the role of endogenous hormone in maintenance of tolerance. If there is such a role, administration of a vasopressin antagonist alone should facilitate the dissipation of tolerance. We found that a V_1-selective antagonist did in fact enhance the rate of loss of tolerance, while a V_2-selective antagonist did not. These results led us to investigate the effect of chronic ethanol ingestion on vasopressin synthesis and release. Acutely, ethanol is believed to inhibit release of AVP from the neurohypophysis, but little is known regarding hormone synthesis and release during and after chronic ethanol exposure. Both mice and rats were used for these experiments. Rats were exposed chronically to ethanol vapor by inhalation, either continuously for 8

days, or intermittently for 12 days. Mice were fed ethanol in a liquid diet. Hypothalamic mRNA was decreased in rats exposed chronically to ethanol by either inhalation method and sacrificed at the time of withdrawal, as measured by Northern blot analysis. In situ hybridization also showed a dramatic decrease in vasopressin mRNA in the supraoptic and paraventricular nuclei of certain of the ethanol-fed mice. The fact that vasopressin mRNA was decreased by chronic ethanol ingestion regardless of the species, method of ethanol administration, or method used to measure mRNA validates the methods and indicates that the effect of ethanol on vasopressin synthesis is quite general. On the other hand, the effect of ethanol on plasma vasopressin levels depended to some extent on the methodology used for ethanol exposure. In the rats treated continuously with ethanol vapor, plasma AVP levels were increased, and levels in the posterior pituitary were decreased, similar to what was observed in dehydrated animals. However, during dehydration, hypothalamic AVP mRNA levels increased, in contrast to the changes seen in the ethanol-treated animals. Thus, ethanol treatment appeared to decrease AVP synthesis and to interfere with the feedback mechanisms regulating hormone synthesis and release. When rats were treated intermittently with ethanol, changes in plasma and pituitary AVP levels were much less pronounced. The data suggest that the continuous ethanol exposure may produce more stress and/or dehydration than the intermittent treatment, and indicate that these factors can confound the results if only plasma vasopressin levels are measured. These interactions may contribute to conflicting findings regarding vasopressin levels in chronic alcoholics.

The finding of decreased vasopressin synthesis after chronic ethanol ingestion at first appears paradoxical, in light of the role of vasopressin in maintaining tolerance. However, it is not necessary for the synthesis of the hormone to be increased in order for it to modulate tolerance. Perhaps more importantly, while it was previously believed that vasopressin was synthesized only in the hypothalamus, more recently vasopressin-containing neurons have been localized in several brain areas, and vasopressin-containing cell bodies have been identified by immunocytochemistry. We have recently identified vasopressin mRNA in the area of the amygdala. It seems likely that brain AVP would be involved in modulating tolerance, and the effects of dehydration and chronic ethanol ingestion on brain AVP synthesis are under investigation.

We have also demonstrated that vasopressin mRNA is present in anterior pituitary and testes, both by Northern blots and in situ hybridization. The synthesis of vasopressin (i.e., mRNA levels) in these tissues appears to be regulated similarly to hypothalamic vasopressin by dehydration and ethanol treatment. Studies are ongoing to evaluate tissue levels of the hormone during these treatments, and to elucidate the normal mechanisms of regulation of vasopressin synthesis and release in these tissues. In particular, we have found that administration of LH or LHRH to rats decreases vasopressin mRNA levels and vasopressin content in the testes, suggesting that gonadotropins may be physiological regulators of testicular vasopressin synthesis.

Significance to Biomedical Research and the Program of the Institute:

The development of tolerance to ethanol allows the intake of large amounts of ethanol by an individual, possibly leading to development of physical dependence and to pathological changes in the CNS and peripheral organs. The finding that vasopressin, a naturally-occurring hormone, as well as vasopressin antagonists, can modulate tolerance, and the understanding of the mechanism of action of the hormone in the CNS, provides an opportunity for development of benign therapies to modify the development and maintenance of tolerance. From a theoretical standpoint, our studies provide support for the hypothesis that tolerance and learning or memory, as CNS adaptive mechanisms, share certain underlying mechanisms. These studies also offer a means both to analyze the biochemical mechanism of action of neurohypophyseal hormones in the CNS, as related to their behavioral effects, and to understand the neurochemical basis of tolerance, as well as learning and memory. Furthermore, these investigations enhance our understanding of the regulation of neurohypophyseal hormone synthesis and release, and the effect of ethanol on this regulation. The mechanisms that regulate extrahypothalamic vasopressin and oxytocin biosynthesis and release, and the possible roles of these hormones in development or maintenance of ethanol tolerance, will be elucidated in our studies.

Proposed Course:

The effect of AVP on c-fos expression will be examined by in situ hybridization and the effect of AVP on Fos proteins will be examined by Western blot analysis and immunocytochemistry. Studies of the effects of acute and chronic ethanol treatment on vasopressin synthesis in hypothalamus and brain will be continued, as will studies of AVP synthesis in peripheral tissues and its physiological regulation. Furthermore, these investigations will be expanded to examine synthesis and release of oxytocin and dynorphin in hypothalamus and brain. Immunocytochemical techniques will be used in combination with in situ hybridization to evaluate brain oxytocin and vasopressin synthesis during chronic ethanol ingestion. To localize the site of action of AVP in maintaining tolerance, site-specific injections in the septal area and hippocampus will be performed.

Publications:

Tabakoff B, Hoffman PL. Tolerance and the etiology of alcoholism: Hypothesis and mechanisms, Alc: Clin Exp Res 1988;12:184-6.

Hoffman PL, Szabó G, Tabakoff B. Vasopressin and alcohol tolerance, Subst Abuse 1987;8(3):3-13.

Hoffman PL, Szabó G, Tabakoff B. The effects of vasopressin and related peptides on tolerance to ethanol, Proc Intl Symp on Peptides and Amino Acid Transport, Belgrade, Yugoslavia, in press.

Szabó G, Tabakoff B, Hoffman PL. Ethanol tolerance is influenced by central vasopressin receptors, Proc 50th Anniv Symp A Szent-Györgi, Szeged, Hungary, in press.

Dave JR, Culp SG, Liu L, Tabakoff B, Hoffman PL. Regulation of vasopressin and oxytocin synthesis in anterior pituitary and peripheral tissues, Adv Alc Subst Abuse, in press.

Hoffman PL, Dave JR, Ishizawa H, Tabakoff B. Molecular biological techniques in alcohol research: Vasopressin and ethanol tolerance, Proc Fourth Congress Intl Soc Biomed Res Alcoholism, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA00705-02 LPPS

PERIOD COVERED

October 1, 1987 - September 30, 1988

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

In Vitro Models for Ethanol Effects on Receptor-Mediated Processes

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

PI:	P. Hoffman	Section Chief	LPPS, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
Others:	C. Rabe	Senior Staff Fellow	LPPS, NIAAA
	P. Rathna Giri	Visiting Associate	LPPS, NIAAA
	F. Moses	Guest Researcher	USP, NIAAA

COOPERATING UNITS (*if any*)

LBC, NIDDKD (J. Daly); Medical College of Toledo (R. McGee); CVRDP, FDA (E.M. DeLorme)

LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

SECTION

Section on Receptor Mechanisms

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

A major focus of our work involves an evaluation of the acute and chronic effects of ethanol in the CNS. However, the brain represents a heterogeneous collection of cell types, and distinction of direct and indirect effects of ethanol can be difficult. In vitro cell culture systems can be used to monitor specific, direct effects of ethanol, for comparison and contrast with results obtained in brain tissue and in vivo. Using a primary culture of cerebellar granule cells, we found that ethanol, at low concentrations, inhibited glutamate (acting at kainate or NMDA receptors)-stimulated cyclic GMP production. Cyclic GMP production stimulated by atrial natriuretic peptide (ANP) was much less sensitive to inhibition by ethanol. The results suggested that glutamate receptor-effector coupling is altered by ethanol. Glutamate stimulates soluble guanylate cyclase in a calcium-dependent manner and preliminary findings indicate that ethanol alters glutamate-dependent calcium flux. These results suggest a new hypothesis for ethanol's depressant effects, and interaction of ethanol with NMDA-coupled calcium channels may also contribute to development of ethanol tolerance and dependence. In PC12 cells, we found that although ethanol stimulated adenylate cyclase (AC) activity in cell membranes, as in brain, ethanol inhibited agonist-stimulated cyclic AMP accumulation in one subclone of intact PC12 cells. In a second subclone ethanol often increased cyclic AMP levels. The inhibition of cyclic AMP production in the first subclone did not depend on Gi or on protein kinase C activity. The results demonstrate that the effect of ethanol on cyclic AMP accumulation depends on the characteristics of AC regulation in various cell types, so that extrapolations to other systems must be cautious. Nevertheless, the use of cell cultures allows investigations that can enhance our understanding of the sites and mechanisms of action of ethanol and of changes in biochemical systems that may, in vivo, be associated with ethanol tolerance and physical dependence.

PROJECT DESCRIPTION

Investigators:

P. Hoffman	Section Chief	LPPS, NIAAA
B. Tabakoff	Scientific Director	NIAAA
C. Rabe	Senior Staff Fellow	LPPS, NIAAA
P. Rathna Giri	Visiting Associate	LPPS, NIAAA
F. Moses	Guest Researcher	LPPS, NIAAA
E. M. DeLorme	Staff Pharmacologist	CVRDP, FDA
R. McGee	Professor	Med. College of Toledo
J. W. Daly	Chief, Laboratory of Bioorganic Chemistry	NIDDKD

Objectives:

The goal of this project is to develop in vitro systems that can be used to model certain aspects of nervous system function, particularly with respect to the function of neurotransmitter and neuromodulator receptors. In vitro cell cultures provide relatively homogeneous systems in which the biochemical as well as physiological responses to receptor activation can be studied in detail in intact cells. These preparations can also be used to evaluate the acute and chronic effects of ethanol on specific receptor-mediated processes under controlled conditions, in systems that mimic the intact physiology of the CNS. Thus, these studies are designed for comparison with studies in brain, to define the specific sites of action of ethanol, and to determine if adaptive changes in various aspects of receptor-effector coupling processes occur at the initial sites of action of ethanol. The findings from these studies can be used to devise experimental protocols that will, in vivo, relate biochemical changes to the development of ethanol tolerance and/or dependence.

Methods:

Cell Cultures. The rat pheochromocytoma cell line, PC12, was grown in plastic tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated horse serum and 5% fetal bovine serum at 37°C in a humidified atmosphere of 90% air: 10% CO_2. Cell viability was assessed by trypan blue exclusion. A subclone of PC12 cells obtained from Dr. John Daly, NIDDKD, was grown in DMEM containing 6% horse serum and 6% fetal bovine serum. Cerebellar neuronal (granule cell) cultures were obtained from eight-day old rats (Wilkins et al., Brain Res. 115: 181, 1976). Cells were plated onto Falcon dishes coated with poly-L-lysine, and were grown in basal modified Eagle's medium containing heat-inactivated fetal calf serum, gentamycin and KCl. The culture dishes were incubated at 37°C in humidified 95% air: 5% CO_2.

After 18-20 hr, cytosine arabinoside was added to the culture medium to inhibit the replication of non-neuronal cells. The culture medium was renewed at days 2, 5 and 8 of culture, and cells were used on day 8 of culture. Cell viability was monitored by ^{51}Cr release. Glial cells were identified by immunofluorescence, using antiserum to glial fibrillary acidic protein.

Neurochemical Measurements in Cultured Cells. Adenylate cyclase activity in PC12 cell membranes was assayed as described previously (Saito et al., J. Neurochem., 44: 1037, 1985) by monitoring conversion of ^{32}P-ATP to ^{32}P-cyclic AMP. Adenylate cyclase activity was measured in intact cells by labeling cellular adenine nucleotides with ^{3}H-adenine, incubating with various agonists, and isolating ^{3}H-labeled cyclic AMP. Cyclic AMP content of intact PC12 cells was measured by radioimmunoassay (New England Nuclear Corp., Boston, MA). Cyclic GMP content of cerebellar granule cells was also determined by radioimmunoassay (New England Nuclear Corp., Boston, MA).

Major Findings:

Ethanol has been shown to stimulate adenylate cyclase activity in cell membrane preparations of peripheral organs as well as brain. In intact cells, however, adenylate cyclase is subject to a variety of regulatory processes, including the influence of other second messenger systems, which may not be operative in membrane preparations. We therefore compared the effects of ethanol on adenylate cyclase (AC) activity in membrane preparations of PC12 cells, and on whole cell cyclic AMP levels. In the presence of guanine nucleotides and agonists, ethanol stimulated AC activity in membrane preparations of PC12 cells in a concentration-dependent manner, similar to previously-reported results in brain cell membranes (Saito et al., J. Neurochem. 44: 1037, 1985). However, when adenylate cyclase activity was measured in intact cells, by quantitating ^{3}H-cyclic AMP accumulation from ^{3}H-labeled adenine nucleotides, ethanol inhibited the accumulation of ^{3}H-cyclic AMP in a concentration-dependent manner. This inhibition was observed in the presence of a phosphodiesterase inhibitor, indicating that ethanol did not act by increasing phosphodiesterase activity. Similarly, when cyclic AMP levels in whole cells were measured, ethanol was found to inhibit the stimulation of cyclic AMP production by adenosine, vasoactive intestinal peptide (VIP) and forskolin. The data indicated that, although ethanol can stimulate adenylate cyclase activity in a membrane preparation, ethanol has other effects in the intact cell that can counteract this action. The inhibition of cyclic AMP production is not due to an interaction with G_i, the inhibitory guanine nucleotide binding protein, since treatment of PC12 cells with pertussis toxin, which blocked the inhibitory action of muscarine, did not affect the inhibitory response to ethanol. Activation of protein kinase C by phorbol esters has been reported to stimulate AC activity in some systems, and to inhibit in others. Therefore, an effect of ethanol on this enzyme system could contribute to changes in AC activity in intact cells. Phorbol esters inhibited cyclic AMP accumulation in PC12 cells, similar to ethanol, suggesting that ethanol might produce its effects by activating protein kinase C. However, when cells were treated with a protein kinase C inhibitor,

staurosporine, the effect of ethanol was not reversed, while that of the phorbol ester was reversed. As another means to evaluate the role of protein kinase C in the actions of ethanol, a subclone of PC12 cells, in which cyclic AMP accumulation was reported to be stimulated by phorbol esters (Hollingsworth et al., FEBS Lett. 196: 131, 1986) was obtained from Dr. John Daly (NIDDKD). Interestingly, ethanol did not inhibit cyclic AMP accumulation in these cells, and frequently had a stimulatory effect (as well as stimulating AC activity in a membrane preparation), although phorbol esters were inactive in our experiments. The data do not provide a clear-cut mechanism by which ethanol inhibits cyclic AMP accumulation in one subclone intact PC12 cells, although an action at G_i or protein kinase C seem to be ruled out. However, the results indicate that stimulation of AC activity in cell membrane preparations does not always lead to increases in cellular cyclic AMP. The response to ethanol in the whole cell appears to reflect the mechanisms regulating cyclic AMP levels in a given cell type, and varies depending on the functional state of AC in the cell. Thus, cell culture systems, particularly those derived from tumors, or transformed cells, do not necessarily provide valid models for the effects of ethanol in the CNS, and measures of the acute in vitro response to ethanol of AC in membrane preparations may not be an accurate reflection of the in vivo situation.

Primary cultures of cerebellar granule cells have been used to evaluate the effects of ethanol on another second messenger system, agonist-stimulated cyclic GMP accumulation. In brain, ethanol has been consistently shown to reduce the levels of cerebellar cyclic GMP, but the mechanism of this action is not clear. We have evaluated the effect of ethanol on stimulation of cyclic GMP accumulation by atrial natriuretic peptide (ANP), which activates particulate guanylate cyclase, and glutamate, which stimulates soluble guanylate cyclase in a calcium-dependent manner. We found that glutamate-stimulated cyclic GMP production was quite sensitive to inhibition by ethanol, with substantial inhibition occurring at 50 mM ethanol. Inhibition was observed whether the response to glutamate was measured in the presence of Mg^{++} (glutamate acting at the kainate receptor) or in its absence (glutamate acting at the NMDA receptor). Ethanol was much less potent at inhibiting the response to ANP. The coupling between the glutamate receptor and guanylate cyclase seems to be most sensitive to ethanol, since ethanol had little effect on basal cyclic GMP levels, and low concentrations of ethanol have been reported to increase, rather than decrease, glutamate binding. Since glutamate stimulation of cyclic GMP production depends on increases in intracellular calcium, we have initiated studies of glutamate-stimulated calcium flux in cerebellar granule cells. Preliminary results indicate that quite low concentrations of ethanol inhibit NMDA-coupled calcium flux, and somewhat higher concentrations inhibit kainate-coupled calcium uptake. These results suggest an that an important site of action of ethanol may be at receptor-operated calcium channels. Inhibition of glutamate receptor-effector coupling may not only be important for the acute, depressant actions of ethanol, but also for tolerance to and physical dependence on ethanol, since NMDA-coupled calcium channels have been postulated to be involved in various other aspects of neuroadaptation, including memory processes and long-term

potentiation.

Significance to Biomedical Research and the Program of the Institute:

Intact cells in culture can provide excellent models for studying the effects of ethanol on receptors-effector coupling processes in brain. Our studies, however, have pointed out some pitfalls in using these systems uncritically, since the functional state of the receptor-adenylate cyclase system in a given cell type can influence the response to ethanol. On the other hand, cells in culture, and particularly primary cultures, can profitably be used to determine basic mechanisms of second messenger regulation, and the sites of action of ethanol in altering this regulation. Data obtained in the relatively homogeneous cells in culture can be applied to the more complex systems in brain. The studies of agonist-stimulated cyclic GMP production have provided a new hypothesis to explain ethanol's acute CNS actions, as well as a possible mechanism for neuroadaptation to ethanol in the CNS.

Proposed Course:

Primary cultures of cerebellar granule cells will be used to characterize the effects of ethanol on kainate and NMDA-stimulated cyclic GMP production, calcium flux, and intracellular calcium levels. In addition, the interactions of ethanol with other drugs that modulate the activity of the glutamate receptor (e.g., glycine, PCP), will be examined in these cells, as will the molecular mechanism by which glutamate stimulates soluble guanylate cyclase. Primary cultures of hippocampal neurons will be developed in which to further elucidate the effect of ethanol on glutamate receptor-effector coupling processes, including the possible role of G proteins in regulation of receptor-operated calcium channels. The studies of cyclic AMP production in PC12 cells are completed.

Publications:

DeLorme EM, Rabe CS, McGee R. Regulation of the number of functional voltage-sensitive Ca^{++} channels on PC12 cells by chronic changes in membrane potential, J Pharmacol Exp Ther 1988;244:838-42.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00464-07 LPPS

PERIOD COVERED

October 1, 1987 - September 30, 1988

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

Ethanol and Cellular Calcium Metabolism

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

P.I.:	H.C. Pant	Research Chemist	LPPS, NIAAA
Others:	J. Shah	Visiting Fellow	LPPS, NIAAA
	M. Virmani	Research Chemist	LPPS, NIAAA

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LAB/BRANCH

Laboratory of Physiologic and Pharmacologic Studies

SECTION

Section on Receptor Mechanisms

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS: 1.9	PROFESSIONAL: 0.9	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.)

Recent studies show that several cell types that mobilize intracellular calcium in response to hormones or neurotransmitters also hydrolyze phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and inositol trisphosphate (IP3), which may mediate the mobilization of intracellular calcium. In the present study, we investigated the involvement of IP3 in calcium mobilization in rat brain microsomes. Ethanol-induced neurotransmitter release in unstimulated synaptosomes was found to be independent of the extrasynaptosomal calcium concentration. Therefore, the effects of alcohol on microsomal calcium uptake and release were also studied. IP3 caused a rapid release of calcium from brain microsomes. In vitro addition of 100 mM ethanol had no effect on ATP-dependent calcium accumulation in the microsomes, but the same concentration of ethanol released 25% of the total accumulated calcium from the microsomes. Ethanol induced calcium release in a concentration-dependent manner over the range of 30 mM to 500 mM. The amount of calcium release increased with higher alcohols. The effect of ethanol was temperature-dependent, suggesting a diffusion-controlled process for calcium transport. These results indicate that the stimulatory effect of ethanol on resting release of neurotransmitters in rat brain may be due to the microsomal release of calcium. This project has been terminated.

Publication:

Shah J, Cohen RS, Pant HC. IP3 induced calcium release in brain microsomes. Brain Res 1988 419: 1-6.

Shah J., Pant HC. Potassium channel blockers inhibit IP3-induced calcium release in brain microsomes isolated from rat brain. Biochem J 1988 250: 617-620.

PHS 6040 (Rev. 1/84)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 AA00400-03 LPPS

PERIOD COVERED

October 1, 1987 - September 30, 1988

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

Selective Breeding for Ethanol Tolerance

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

PI:	K.A. Grant	Staff Fellow	USP, NIAAA
	B. Tabakoff	Scientific Director	NIAAA
Other:	R.M. Werner	Veterinarian	DICBR, NIAAA
	P.L. Hoffman	Section Chief	LPPS, NIAAA

COOPERATING UNITS *(if any)*

VRB, SASA (C. Hansen, W. Jackson, N. Watson);
BIOCON (B. Till)

LAB/BRANCH

Unit for Special Projects

SECTION

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, Maryland 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

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

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

The development of chronic tolerance to the motor disrupting effects of ethanol has been studied in genetically heterogeneous rats obtained from the N:NIH stock. The preliminary data demonstrate that the development of tolerance was widely distributed and unimodal in this population of rats. In addition, there was no sex differences in the amount of tolerance developed. Thus, the measure of tolerance chosen and the procedure used to develop tolerance with this population of genetic-heterogeneous rats appears to fulfill the necessary requirements to begin a successful breeding program. Rats from the original gene pool will be selectively breed to form divergent lines of rats based on the degree of functional tolerance obtained after an eight day ethanol exposure period. Three lines of rats will be developed: a line which develops a large amount of functional tolerance to ethanol, a line which develops littler or no functional tolerance to ethanol, and a control line which is randomly mated, irrespective of their tolerance development. Each line will be composed of ten families to minimize rapid inbreeding. It is also proposed that replicates of each trait will theoretically contain all alleles associated with the trait, while alleles not associated with the trait will be randomly distributed. Thus, these animals will be a resource for investigators interested in neurobiological and behavioral correlates of chronic functional tolerance to ethanol.

PROJECT DESCRIPTION:

K. Grant	Staff Fellow	USP, NIAAA
B. Tabakoff	Scientific Director	NIAAA
P. L. Hoffman	Section Chief	LPPS, NIAAA
R. M. Werner	Veterinarian	DICBR, NIAAA
N. Watson	Chief	VRB, SAS
W. Jackson	Deputy Chief	VRB, SAS
C. Hansen	Geneticist	VRB, SAS
B. Till	Lab Technician	BIOCON

Objectives:

The goal of this project is to breed selected lines of rats that differ in their ability to develop functional tolerance to ethanol. The selection process will theoretically result in a separation of all the alleles related to developing a high degree of tolerance from those alleles related to a developing a low degree of tolerance. Alleles which are not related to the development of tolerance are expected to remain randomly distributed in both lines of rats. Therefore, biochemical traits that differ between the selected lines will be related to the ability to develop tolerance to ethanol. The availability of these animals will be an invaluable tool in the study of neurobiological correlates of ethanol tolerance. In addition, the animals will be helpful in determining if other behavioral effects of ethanol, such as the reinforcing properties, are manifestations of these alleles.

Methods:

The original foundation stock for this breeding project will be the heterogeneous N:NIH stock. These animals have been tested for their development of chronic tolerance to ethanol, and appear to be appropriate for beginning a selective breeding project (Tabakoff and Culp, Alc: Clin. Exp. Res. 8:495, 1984; Grant et al., Alc: Clin. Exp. Res., in press). The number of lines bred will be three: a high tolerant line, a low tolerant line and a random control line. In addition, replicates of these lines will be bred. The breeding scheme used will be one that minimizes inbreeding, using 10 families per line and an cross-family mating procedure that avoids common parents and grandparents.

Major Findings:

Using the concentration of ethanol in the blood at regain of aerial righting reflex (ARR) as a measure of ethanol's motor disrupting effects, tolerance developed after 8 days of chronic ethanol exposure has been studies in rats from the N:NIH stock. Some rats developed a great deal of tolerance, showing an increase of over 250 mg% in BEC at regain of ARR, while other rats had decreases in BEC at regain of ARR. There were no gender differences in the amount of chronic tolerance based on the difference in BEC at regain of ARR. The distribution of tolerance development was wide and unimodal, reflecting a large amount of genetic heterogenity with regards to this trait.

Significance to Biomedical Research and the Program of the Institute:

The development of tolerance to ethanol allows the intake of large amounts of ethanol by an individual, possibly leading to development of physical dependence and to pathological changes in the CNS and peripheral organs. The development of lines of animals with differing ability to develop ethanol tolerance will provide a model in which neurobiological

correlates of ethanol tolerance can be studied. These animals will provide a resource for all investigators interested in the determinants of ethanol tolerance. Understanding the biochemical basis for functional tolerance can eventually lead to therapies that can modify the development or extent of tolerance.

Proposed Course:

Studies are underway typing the families from the N:NIH stock of rat to assign the initial families in each line. The breeding project is due to begin in late summer, with approximately 3 generations/year selectively bred. At the 5th generation, a major evaluation of the separation between line will take place. Other studies are being conducted with the N:NIH stock of rats to determine the contribution of acute tolerance development to the amount of chronic tolerance developed to the effects of ethanol.

Publication:

Grant KA, Hoffman PL, Tabakoff B. Neurobiological and behavioral approaches to tolerance and dependence. In: Nature of Dependence, Oxford University Press, in press.

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