Administrative Document

Annual Report of Intramural Research Program Activities

National Institute on Alcohol Abuse and Alcoholism

Fiscal Year 1985

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, 1984 to September 30, 1985

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, MD 20205

RC
565
N2772
1985

TABLE OF CONTENTS

* Includes all projects active in FY 1984 and FY 1985, some of which have been consolidated or terminated during FY 1985. See individual project reports for status.

Annual Report of the
Division of Intramural Clinical and Biological Research
National Institute on Alcohol Abuse and Alcoholism
October 1, 1984 to September 30, 1985
Boris Tabakoff, Ph.D., Director

The NIAAA Intramural Research Program (IRP) is dedicated to the generation of knowledge regarding the acute and chronic effects of alcohol on various organs and on the pathophysiologic or behavioral consequences of alcohol abuse. The aims of the IRP are to develop new methods for the diagnosis, treatment, and prevention of alcohol-related problems. To understand ethanol's effects on the human organism, the Program is also generating basic knowledge of the systems that control physiologic function and behavior. The IRP became fully established with the opening of the Laboratory of Clinical Studies' clinical research ward in January 1984. I began my tenure as Director of the DICBR in October 1984.

In this first Annual Report as Director of the Intramural Research Program, I wish to give thanks for the help that was provided by colleagues within NIH, ADAMHA, and the staff of NIAAA who made my transition to this administrative role pleasant as well as efficient. Assuming the Directorship of the IRP was made even more challenging by the necessity to perform the duties of Acting Deputy Director for the Institute during several months of this fiscal year. Special thanks go to Laura Rosenthal, Deputy Director, DICBR, who not only indoctrinated me into the workings of government research programs but also provided outstanding support in managing the administrative functions of the program throughout the year. Dr. Robert Niven, Director, NIAAA, recruited me into the NIAAA and continued to provide the moral support necessary to establish the Office of the Director, DICBR, and to appropriately manage the Program. My thanks are also extended to the other Scientific Directors of the NIH and ADAMHA Intramural Research Programs who welcomed me on campus and into the NIH/ADAMHA intramural research community. Drs. Gruelich (NIA), Roth (NIADDK), Goodwin (NIMH), and Jaffe (NIDA) made special efforts to help me and the NIAAA IRP. Finally, the guidance provided by Dr. Rall, Deputy Director for Intramural Research, Dr. Chen, Associate Director for Intramural Affairs, and Dr. Becker, Associate Director for Research Services, was most valuable in generating knowledge and appreciation of how the NIH research community functions.

My initial year as Director of the Intramural Research Program has been sometimes difficult but most times exciting and rewarding. Despite severe initial constraints on the program in terms of personnel, space, and resources, the Intramural Research Program has prevailed and has produced outstanding research. This is attested to by the quantity and quality of the publications of the Program's scientists, the honors bestowed on them, and the multitude of lectures they have been invited to present at national and international meetings. Major talks by the intramural staff were integral parts of the ACNP meetings in Puerto Rico, the Traffic Safety meetings in Australia, the meetings on the Biology of Alcoholism in the Soviet Union, and the Biological Psychiatry meetings in Philadelphia, to name a few. In addition, Markku Linnoila, Chief of the Laboratory of

Clinical Studies, presented the NIH Medicine for the Layman lecture dealing with alcohol abuse and alcoholism.

The accomplishments of the Program were also recognized by the review of the Laboratory of Clinical Studies by the Board of Scientific Counselors. The Board, headed by Dr. Harold Kalant, was not only laudatory but provided excellent guidance for further enhancing the scientific endeavors of the Intramural Research Program.

The members of the Board of Scientific Counselors are:

Harold Kalant, M.D., Ph.D	- Professor of Pharmacology, University of Toronto
Thomas Boll, M.D.	- Director, Medical Psychology Program, University of Alabama
Dora Goldstein, M.D	- Professor of Pharmacology, Stanford Medical School
Dan Lindsley, Ph.D.	- Professor of Biology, University of California, San Diego
Alton Meister, M.D.	- Chairman, Department of Biochemistry, Cornell University
Stanley Rapoport, M.D.	- Chief, Laboratory of Neurosciences, National Institute on Aging
Hyman Zimmerman, M.D.	- Professor of Medicine and Director of Gastroenterology, George Washington University Medical Center

Meetings of the Scientific Counselors for the purpose of reviewing the Laboratory of Preclinical Studies and the Laboratory of Metabolism are scheduled for fiscal year 1986.

Not only did the DICBR fully establish the process for review of its scientific endeavors, it also established review procedures for staff promotions and tenure that are consistent with NIH standards. The IRP Animal Care Committee continued to function admirably in reviewing research involving animals consistent with recommendations of the American Association for the Accreditation of Laboratory Animal Care. The Program also established a Safety Committee, whose Chairman participates with the Chairmen of safety committees of NIH Institutes in establishing safety guidelines and preventing and rectifying unsafe laboratory practices.

During this last year the new Laboratory for Studies of Neuroadaptive Processes was established as part of the Intramural Research Program. Because of space constraints, the personnel of this Laboratory were distributed between three locales. In addition to space on the NIH campus and at the DANAC Building in Rockville, MD, a portion of the Laboratory is housed at the NIDA Addiction Research Center in Baltimore. Although some communication problems have arisen between the sections of this newly established Laboratory, the personnel have been provided excellent opportunities for collaboration with the staff of NIDA and NIA at the Baltimore Francis Scott Key Medical Center. In addition, new space for the

outpatient program of the Laboratory of Clinical Studies was acquired within the Ambulatory Care Research Facility at the NIH. This space will allow the initiation and expansion of a number of important protocols involving subjects and procedures that were unmanageable in facilities previously available to the IRP. Research space for the Laboratory of Preclinical Studies is currently under renovation. When completed, these changes will provide appropriate conditions for the sophisticated electrophysiological experiments being performed by the scientists in this laboratory.

During my initial year as Scientific Director of the NIAAA Intramural Research Program I have made a commitment to enhance its integration into the NIH research community. In addition to establishing administrative procedures consistent with NIH norms, the IRP has cooperated with other NIH Institutes to support the establishment and development of common research resources on the Bethesda campus. The availability of a cyclotron and PET scanners is an invaluable commodity for research protocols investigating the functional anomalies of alcohol induced organic brain damage and other forms of alcohol dementia. Our financial contribution to the purchase and installation of a cyclotron on the NIH campus was more than compensated by the availability of PET technology to the NIAAA intramural scientists. Our scientists in turn are collaborating with other scientists on the NIH campus to establish means for correlating structural and functional information about the brain derived from the use of CT, PET, and NMR technology. In fact, the NIAAA Intramural Research Program embarked on a major initiative to institute NMR technology as part of the research resources available to our scientists. With the help of Dr. Britton Chance of the University of Pennsylvania School of Medicine, a committee of experts was gathered to review the relevant research portfolio of the Program and to advise on the necessity, feasibility, and means for introducing NMR technology into its research program. The advice of this panel has encouraged activities in a number of areas including the participation of Richard Veech and myself in the planning for an in vivo NMR research facility on the NIH campus. This collaborative venture between the various institutes of NIH and ADAMHA will be another example of how collaboration can provide research opportunities even at times of scarce research resources.

Although the ability to recruit new personnel to the Intramural Research Program has been quite limited, a positive note within the area of limited personnel resources was the announcement this past year by the NIAAA Division of Extramural Research encouraging young scientists to apply for fellowships to work in the NIAAA Intramural Research Program. There are a number of outstanding scientists in the Program who would make excellent preceptors for training individuals in various aspects of alcohol-related research, and this fellowship program will provide an excellent opportunity to enhance the number of well-trained individuals in the alcohol research community.

The Intramural Research Program has sponsored and cosponsored a number of outstanding seminars and workshops during this last year. For example, the Office of the Scientific Director has coordinated the NIAAA portion of the ADAMHA Administrators Research Forum. Some of the scientists invited to

make presentations at this forum were Drs. Dora Goldstein, Stanford University; Emanuel Rubin, Hahnemann University; Barry Hoffer, University of Colorado; and Henri Begleiter, Downstate University of New York. The Intramural Research Program, with the Division of Extramural Research, cosponsored and participated in a symposium on the use of imaging techniques in alcohol research held at the Research Society on Alcoholism's annual meeting.

During the year the Scientific Director and other IRP staff made a number of scientific presentations to legislative and administration staff. The participants in these presentations ranged from George Keyworth, Presidential Science Advisor and William Roper, Special Assistant to the President for Health Policy, to members of the Office of the Assistant Secretary for Health, and members of Congress who are concerned with the issues surrounding the health and social consequences of alcohol abuse. The Office of the Scientific Director has also been active in various educational functions with members of the press and with foundations supporting alcohol research. Through such activities information about the achievements of the Intramural Research Program are made available to the public and enthusiasm for support of such research is enhanced. It is, after all, a major concern of the Scientific Director to encourage the most outstanding research and to generate an understanding of the needs and benefits of these endeavors.

It is my privilege to have become associated with the group of fine scientists in the NIAAA Intramural Research Program and especially the Chiefs of the Laboratories -- Markku Linnoila of the Laboratory of Clinical Studies, Forrest Weight of the Laboratory of Preclinical Studies, and Richard Veech of the Laboratory of Metabolism. The accompanying reports in this document, describing the research achievements of the intramural laboratories, attest to the reasons for my enthusiasm about the accomplishments of the past year and the outlook for the future.

Annual Report of the Laboratory of Clinical Studies
National Institute on Alcohol Abuse and Alcoholism
October 1, 1984 to September 30, 1985
Markku Linnoila, M.D., Ph.D., Chief

Introduction

During fiscal year 1985, investigators in the Laboratory of Clinical Studies continued the longitudinal projects outlined in the 1984 annual report and started new projects in the areas of violence, suicide, and depression in alcoholics. The occupancy rate of the 10-bed research ward has continuously exceeded 70 percent, which is close to a practical maximum. The Laboratory will obtain permanent outpatient space by the end of July 1985, and its animal space is being renovated.

The Laboratory is investigating inheritance, family system effects, central nervous system and liver effects, and other complications of alcohol abuse and alcoholism. It is also testing novel approaches to the treatment of alcoholism and its complications.

1. Clinical Science

The clinical science program is equipped for 24-hour electroencephalographic (EEG) telemetry, EEG sleep studies, and 24-hour activity and body temperature monitoring. Feelings and behaviors of the patients are assessed by the patients as well as by trained nurses, who do not know which experimental therapies apply to which patient. For the first time in alcohol research, these methods are bringing together advanced, objective, quantitative, and continuous measurements of physiology and behavior with sophisticated biochemical, brain imaging, genetic, and family studies. The main objectives of the research on the ward are:

1. Description of the prolonged and insidious withdrawal syndrome, which follows acute withdrawal in alcoholics.

2. Elucidation of physiological functions in patients with alcohol amnesic syndrome and with alcohol-induced dementia.

3. Testing new treatments to ameliorate symptoms of chronic alcohol-induced brain disease such as memory deficits.

4. Behavioral and biochemical description of depression and suicidal behavior in alcoholics.

The main findings are that patients with Korsakoff's psychosis have rapid-eye-movement sleep anomalies similar to those seen in major depression, and that children of familial alcoholics show a lower k_m of transketolase for thiamine.

The Outpatient Clinic screens prospective patients and family members and follows up on patients in long-term treatment protocols. Because alcohol dependence has a hereditary component and because the risk of becoming alcohol dependent is exacerbated by social stressors, the Laboratory is

examining the relationship of the following independent variables to the length of abstinence in alcoholics:

1. New pharmacological maintenance treatments, mainly neurotransmitter-specific antidepressant drugs and transmitter precursors, some of which alter consumption of alcohol in animals and acute effects of alcohol in healthy humans;

2. Specific group and psychotherapeutic interventions.

A clientele of about 100 alcoholics is expected to be in followup by the end of calendar year 1985.

2. Family Studies

The family studies program has recruited more than 60 families which contain alcoholic family members for detailed physiological and biochemical studies and comparison with age- and sex-matched controls. To identify risk and protective factors for the development of alcohol dependence, particular attention is being paid to longitudinal study of the children of alcoholics. During the followup, behavioral, physiological, and pharmacological challenges are being administered repeatedly to index and control subjects. It is hoped that this strategy will further elucidate variables associated with the risk of becoming an alcoholic.

The main findings are a very high incidence of accidents, suicide attempts, and other forms of violence in middle-class families of alcoholics.

3. Genetic Studies

Because of the strong genetic contribution to the risk of becoming alcohol dependent, work in the area of genetics involves the conduct of linkage and association studies in patients, particularly members of intensively studied families with multigenerational alcoholism and male alcoholics who are tested for impulsivity. Cell lines are established, and DNA and protein polymorphisms are used as genetic markers. Specifically bred mouse strains showing differences in behavioral sensitivity towards ethanol are analyzed to assist in identifying loci that participate in determining such sensitivities. Furthermore, the investigators are studying alcohol and aldehyde dehydrogenases using monoclonal antibodies, enzyme methods, novel protein electrophoretic methods, and DNA probes in order to better understand their structural and functional variants and their evolutionary origins.

A new method for producing affinity columns for alcohol dehydrogenases is being sent for patent review.

Because violent behavior is prevalent among intoxicated alcoholic men and is associated with structural abnormalities of the Y chromosome, genetic studies will specifically elucidate chromosome markers associated with this behavior. Probes for genes located on the Y chromosome will be obtained from other laboratories and developed on site. These studies are expected to run for several years, but they are potentially of great importance if they succeed in identifying genetic factors that control violent behavior in alcoholics and problem drinkers.

4. Clinical Brain Research

Investigators in the area of clinical brain research conduct sophisticated electrophysiological, neuropsychological, and brain imaging studies on alcoholics, individuals at high risk of developing alcoholism, heavy and light social drinkers, 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 produced by alcohol in traffic and occupational tasks will be tested.

New strategies to reverse the cognitive deficits associated with acute alcohol intoxication and with chronic alcoholism are being explored.

5. 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 effects on impaired liver function on pharmacokinetics and pharmacodynamics in alcoholics. These studies are expected to provide rationales for individualized drug dosage in treating 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. Theoretically, the use of appropriate drugs should allow quantifying both liver blood flow and enzyme activity from breath tests.

Pharmacokinetic principles and clearance concepts are being applied to the disposition of neurotransmitters and their metabolites in humans in order to describe their disposition in vivo. The goal is to define new testable hypotheses about the biochemical bases of alcoholism 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 measuring concentrations of norepinephrine and its metabolites in various body compartments.

6. Analytical Chemistry

Work in the area of analytical chemistry concerns the measuring of prostaglandins, leukotrienes, and other oxygenated fatty acid metabolites in cerebrospinal fluid from alcoholics and healthy volunteers. Investigators are also studying biological membranes in alcoholics to evaluate changes in the species and molecular arrangements of phospholipids comprising those membranes. These aspects of lipid metabolism and phospholipid function are known to be particularly sensitive to perturbances by alcohol.

Further progress has been made in the structural characterization of a novel series of leukotriene-like compounds formed in the central nervous system. The synthesis of these hydroxylated decosanoids is stimulated by low doses of ethanol. In testing the effects of ethanol in the cardiovascular system it was found that altered contractility of the heart, and blood pressure, after ethanol exposure may have a common etiology in the disruption of

prostanoid metabolism. These studies may eventually explain the association between a low incidence of atherosclerotic disorders and moderate consumption of alcohol and the increased incidence of cardiovascular disorders and stroke in alcoholics.

7. Neurochemistry

A program of research in neurochemistry is providing logistical and practical support for clinical receptor function and neuropeptide studies in alcoholic patients, individuals at high risk of developing alcoholism, and healthy controls. The program is conducting animal experiments and in vitro cell culture studies designed to elucidate the effect of alcohol on cellular functions, from membrane receptors to rates of genomic transcription. These studies are expected to provide rationales for new pharmacological treatments of alcoholism.

Regular section and unit chief meetings have been held, a guest lecturer program involving distinguished investigators in alcoholism research is operational, and the laboratory library functions well. Thus the basic structure to support the comprehensive clinical research program on the causes and complications of alcoholism outlined above has been strengthened in 1985. Information on improving prevention and treatment of alcoholism and its complications is accumulating rapidly.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00246-02 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Measurement of Norepinephrine and its Metabolites in Various Body Compartments

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

PI:	S. Guthrie	Senior Staff Fellow	LCS, NIAAA
Others:	E. Lane	Staff Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	I. Parashos	Visiting Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*
Section of Clinical Pharmacology, LCS, NIMH (M. Rudorfer, W. Potter)

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
0.8	0.6	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.)*

Attempts to characterize the neurotransmitter systems in humans have focused on quantification of the neurotransmitters seronin (5HT), norepinephrine (NE), dopamine (DA), and several metabolites of these monoamines in various body fluids (urine, plasma, CSF). These neurotransmitter systems are suspected to be functioning abnormally in a variety of disorders (e.g., alcoholism, depression, schizophrenia). In order to understand the meaning of measuring concentrations of neurotransmitters and their metabolites, it is necessary to understand their pharmacokinetics in different body compartments. Plasma and CSF concentrations, as well as 24-hour urinary measures of NE, DA, and 5HT and their metabolites (VMA, MHPG, NM, 5HIAA, HVA) were collected in placebo-treated, depressed subjects, alcoholics, and healthy volunteers. Samples were also collected in depressed subjects following treatment with antidepressants. Utilizing these data, a model was formulated which described the disposition of MHPG. Rat liver perfusion using two NE metabolites, NM and MHPG, will be used to determine the fraction of a metabolite being transformed by each of several metabolic pathways. A better understanding of these relationships will help identify differences in formation, metabolism, and elimination of these neurotransmitters in humans. This will offer insight into the functioning of these systems and into the abnormalities of functioning that result in the previously observed abnormal concentrations of these neurotransmitters and their metabolites in certain disease states.

PROJECT DESCRIPTION:

Investigators:

S. Guthrie	Senior Staff Fellow	LCS, NIAAA
E. Lane	Staff Fellow	LCS, NIAAA
I. Parashos	Visiting Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
M. Rudorfer	Senior Staff Fellow	LCS, NIMH
W. Potter	Section Chief	LCS, NIMH

Objectives:

Many studies have suggested abnormalities of various neurotransmitter systems in a variety of disease states. Many of these reports have been based on quantification of a neurotransmitter and its metabolites in various body fluids at a single point in time. The concentrations of neurotransmitters (5HT, NE, DA) and metabolites (MHPG, VMA, 5HIAA, HVA, NM) were determined in a population of depressed, placebo-treated subjects and in a small group of depressed patients being treated with antidepressants. Analysis of these concentrations in different body compartments at equilibrium will help delineate the interrelationship of the neurotransmitter systems as well as help better define any abnormalities of neurotransmitter metabolism present in depression. Rat liver perfusion with NM and MHPG will enable us to determine the fraction biotransformed by each of several different metabolic pathways.

Methods Employed:

(1) Depressed subjects were housed on the Clinical Research Ward at the NIMH and placed on a low monoamine diet. After a minimum period of 3 weeks of placebo or antidepressant treatment, two to seven 24-hour urine collections were made and analyzed for 5HT, 5HIAA, DA, HVA, NE, NM, MHPG, and VMA. Baseline lumbar punctures were performed, and CSF was analyzed for MHPG, NE, 5HIAA, and HVA. Plasma concentrations for baseline values of NE and MHPG were also obtained. Urinary determinations were performed by Dr. F. Karoum's Laboratory at St. Elizabeth's Hospital, while plasma and CSF analyses were done in the Laboratory of Clinical Studies, NIAAA. Statistical analysis of results was accomplished with IBM 370 and DEC 10 computers at the NIH, utilizing the Statistical Analysis System and Bright programs. The analyses of samples from healthy volunteers and alcoholics housed on the LCS, NIAAA Research Ward have not been completed yet. (2) Rats will be sacrificed, using ketamine and ether, the peritoneum will be opened, and the portal veins will be catheterized and connected to an organ perfusion pump. The inferior venacava will also be catheterized to collect the hepatic outflow. The liver will then be perfused with [^{3}H]-NM or [^{3}H]-MHPG in a 20% suspension of outdated human red blood cells, albumin, and Kreb's Ringer bicarbonate buffer solution, saturated with 5% CO_2/95% O_2. The [^{3}H]-NM or [^{3}H]-MHPG and their metabolites will then be spotted on to a silica gel TLC plate impregnated with sodium tetraborate, and developed in a mobile phase of n-butanol/ethanol/tris (2:1:1) for 13 hours. Chromatograms will be visualized with a ferric chloride/potassium ferricyanide spray, and the separated compounds will then be scraped into a liquid scintillation vial and radioactivity will be quantified with a scintillation counter.

Major Findings:

(1) Our analysis, using CSF concentrations and urinary output measurements of MHPG, provides a means of calculating the percentage change in the rate of synthesis of MHPG in the CNS resulting from pharmacological manipulations in humans. Our results suggest that the antidepressant drugs desipramine and zimelidine decrease the synthesis of MHPG in the CNS. (2) There are no findings at this stage.

Significance to Biomedical Research and the Program of the Institute:

Because NE in various body compartments is in disequilibrium, its metabolites may better reflect the overall activity of this neurotransmitter system. The significant correlation between plasma and CSF MHPG concentrations previously reported by other investigators was not replicated in our more extensive data base. Our results appear to reflect dissimilar rates of MHPG formation in individual patients. This would be in agreement with the hypothesis that the activity of the norepinephrine neurotransmitter system is variable in diagnostically heterogenous depressed patients. Our findings of a decreased rate of MHPG formation in the CNS during treatment of depressed subjects with antidepressant drugs agrees with our previous findings of reduced NE turnover, determined by urinary MHPG excretion rates, during antidepressant drug therapy. These findings suggest that the overall effect of these treatments may be to increase the efficiency of NE transmission rather than the rate of synthesis of NE. A better understanding of the metabolism of some of the intermediate metabolites of NE, such as NM and MHPG, will further our understanding of NE disposition. This will help describe any abnormalities of this process that may be present in such disease states as alcoholism and depression.

Proposed Course:

(1) The majority of our data concerning the dopamine and serotonin systems is in the process of being analyzed. Knowledge of the interrelationships of all three neurotransmitter systems may further our understanding of abnormalities in neurotransmitter activity present in depression. Similar determinations of neurotransmitter and metabolite concentrations in different body compartments of alcoholic patients housed on the NIAAA Research Ward will help define abnormalities in activity of neurotransmitter systems that may be unique for this group. (2) Rat liver perfusion will be performed as soon as the techniques of liver perfusion and TLC assay are fully and reproducibly operational.

Publications:

Linnoila, M., Guthrie, S., Lane, E.A., Karoum, F., Rudorfer, M., and Potter, W.Z.: Clinical studies on norepinephrine metabolism: How to interpret the numbers. Psychiatry Research (in press).

Linnoila, M., Lane, E.A., Guthrie, S., Parashos, I., Rudorfer, M., and Potter, W.Z.: CSF, plasma and urine: What do concomitant measurements of norepinephrine and its metabolites mean? Psychopharmacol Bulletin (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00238-03 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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
	R. Eskay	Research Physiologist	LCS, NIAAA

COOPERATING UNITS *(if any)*

VA Medical Center, Washington, DC (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	0.2	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☒ (a2) Interviews

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

Severity of withdrawal symptoms from alcohol was quantified in alcoholics admitted to the Neurology Ward of the Washington, DC Veterans' Administration 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. A manuscript on the norepinephrine findings is in press in Archives of General Psychiatry. We are continuing this work to try 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 with concentrations of norepinephrine and MHPG in the CSF and with the severity of withdrawal symptoms in our patients.

PROJECT DESCRIPTION:

Investigators:

M. Linnoila	Chief	LCS, NIAAA
J. Yergey	Senior Staff Fellow	LCS, NIAAA
R. Eskay	Research Physiologist	LCS, NIAAA
J. Hawley	Neurologist	VA Medical Center

Objectives:

Many symptoms of alcohol withdrawal are indicative of sympathetic 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, DC Veterans' Administration Hospital where the patients are treated and studied under a 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 lumbar punctures on every subject, one early and 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 and neuropeptides with radioimmunoassays.

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 samples 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 correlated 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 a 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:

Hawley, R.J., Major, L.F., Schulman, E.A., and Linnoila, M.: Cerebrospinal fluid MHPG and NE in alcohol withdrawal: correlations with clinical signs. Arch. Gen. Psychiatry (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00256-01 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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)*

Section of Clinical Pharmacology, LCS, NIMH (W. Potter, I. Mefford); Laboratory of Chemistry, NIADDK (K. Kirk, K. Jacobson).

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	0.2	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

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 in human and nonhuman primate cerebrospinal fluid and histamine turnover in various nuclei of the rat brain.

PROJECT DESCRIPTION:

Investigators:

M. Linnoila	Chief	LCS, NIAAA
K. Kirk	Research Chemist	LC, NIADDK
K. Jacobson	Staff Fellow	LC, NIADDK
I. Mefford	Senior Staff Fellow	LCS, NIMH
W. Potter	Section Chief	LCS, NIMH

Objectives:

To develop quantitative methods for the measurement of putative neurotransmitters and their metabolites in femtomolar concentrations.

Methods Employed:

Specific amine reagents with or without electroactive functional groups are used to derivatize monoamine neurotransmitters and their methylated metabolites. The derivatization products are extracted into organic solvents, separated from other similar compounds with liquid chromatography, and quantified with electrochemistry. The methods have been applied to measure serotonin in cerebrospinal fluid and histamine turnover in rat brain nuclei.

Major Findings:

We can reliably and routinely quantify serotonin in human lumbar cerebrospinal fluid and histamine turnover in various nuclei of the rat brain. Studies on the effects of various pathological conditions and pharmacological manipulations on cerebrospinal fluid serotonin concentrations and histamine turnover in the central nervous system are in progress.

Significance to Biomedical Research and the Program of the Institute:

Cerebrospinal fluid serotonin concentrations and histamine turnover in discrete areas of the rat brain have been difficult to quantify in the past. The availability of new and relatively simple methods to quantify these variables will facilitate the understanding of the physiological and pathophysiological significance of neurone systems that use serotonin or histamine as transmitters.

Proposed Course:

We will apply the new assays to study cerebrospinal fluid biochemistry in violent offenders, alcoholics, and patients with alcohol-induced organic brain syndromes, as well as to study effects of ethanol on histamine turnover in distinct areas of the rat brain.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00257-01 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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
Other:	H. Moss	Medical 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	

CHECK APPROPRIATE BOX(ES)

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

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 in control children matched by age, sex, and past alcohol exposure. 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
H. Moss	Medical Staff Fellow	LCS, NIAAA

Objectives:

We wanted to investigate 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:

Nine sons and eight daughters of familial alcoholics and 15 matched control children received i.v. infusions of TRH while at bed rest in our Outpatient Clinic. Triiodothyronine, thyroxine, and TSH concentrations were quantified.

Major Findings:

Sons, but not daughters, of familial alcoholics had markedly higher TSH responses to TRH than their matched controls.

Significance to Biomedical Research and the Program of the Institute:

This is 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.

Proposed Course:

We are quantifying growth hormone and prolactin concentrations, in addition to TSH, to elucidate neurochemical bases for our findings. We are entering the subjects into a longitudinal study and are enlarging our population sample.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00258-01 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

COOPERATING UNITS *(if any)*

Department of Psychiatry, University Central Hospital, Helsinki, Finland (M. Virkkunen); IRP, National Institute of Mental Health, Bethesda, MD (F. Goodwin, L. Brown).

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	0.2	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☒ (a2) Interviews

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

We have investigated neurotransmitter metabolites and glucose metabolism in incarcerated violent offenders, arsonists, and healthy volunteers. We have found that low cerebrospinal fluid 5-hydroxyindoleacetic acid (5HIAA) concentrations and hypoglycemias during oral glucose tolerance tests are associated with impulsive violent acts and fire setting.

PROJECT DESCRIPTION:

Investigators:

M. Linnoila	Chief	LCS, NIAAA
M. Virkkunen	Senior Lecturer	University of Helsinki
F. Goodwin	Director	IRP, NIMH
L. Brown	Staff Psychiatrist	BPS, IRP, NIMH

Objectives:

To investigate biological 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. Oral glucose tolerance tests and MMPIs have been administered to the subjects. Careful forensic psychiatry examinations have been performed on the subjects.

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.

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.

Proposed Course:

We are planning to start to obtain skin biopsies in future subjects to relate the described findings to possible Y-chromosome abnormalities. We will enlarge our sample (which currently consists of more than 60 subjects) and, providing the findings remain the same, will investigate effects of relatively specific serotonin reuptake antagonists in habitually violent patients.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00230-03 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of a Thiamine Deficiency Model of Korsakoff's Psychosis

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

PI:	P. Martin	Visiting Scientist	LCS, NIAAA
Others:	M. Eckardt	Section Chief	LCS, NIAAA
	D. Goldman	Unit Chief	LCS, NIAAA
	E. Lane	Staff Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*
Human Genetics Branch, NICHD (A. Mukherjee)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Science

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

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.)*

Sprague-Dawley rats treated with a thiamine-deficient (TD) diet develop a syndrome that behaviorally and neuropathologically resembles Wernicke's encephalopathy (the acute phase of Korsakoff's psychosis). Rats exhibiting the syndrome of TD improve behaviorally within hours after intraperitoneal injection of thiamine; after 2-3 months, they weigh the same and appear no different from control animals. Recovered animals of both sexes (at least 6 months post-TD) are significantly less sensitive to the effects of ethanol as measured by behavioral impairment and hypothermia, perhaps due to increased alcohol metabolism and/or reduced CNS sensitivity. Therefore, TD may contribute to both the pharmacokinetic and pharmacodynamic tolerance to alcohol observed in chronic alcoholics. Preliminary findings suggest that a similar phenomenon may be operative in rats made thiamine-deficient in utero. Since the duration of time required to develop the syndrome of TD varies greatly in outbred strains, we evaluated 10 inbred rat strains to determine their sensitivities to TD. A TD-sensitive strain (M520) and a TD-resistant strain (F344) were selected for further study. M520 rats metabolized ethanol more rapidly, were less impaired at equivalent blood ethanol levels (BEC), and preferred and drank 10-15 times more ethanol in a free-choice (ethanol solution/water) paradigm than F344 rats. Catalase activity was greater in M520 rats, but liver alcohol dehydrogenase (ADH) activities were not significantly different. After 7 weeks of TD, M520 rats showed a greater reduction of baseline body temperature and peak BEC than control rats of the same strain. Both strains showed a twofold increase in liver ADH during TD, but no significant change in catalase, compared with control rats of the same strains.

PROJECT DESCRIPTION:

Investigators:

P. Martin	Visiting Scientist	LCS, NIAAA
G. Bone	Medical Staff Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
D. Goldman	Unit Chief	LCS, NIAAA
G. Impeduglia	Visiting Fellow	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA
E. Lane	Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
R. Lister	Visiting Fellow	LCS, NIAAA
E. Majchrowicz	Research Chemist	LPS, NIAAA
C. Marietta	Physiologist	LPS, NIAAA
P. Rathnagiri	Visiting Fellow	LCS, NIAAA
N. Salem	Research Chemist	LCS, NIAAA
A. Mukherjee	Section Chief	HGB, NICHD

Objectives:

To evaluate: (1) the contribution of thiamine deficiency to the complications of chronic exposure to ethanol, specifically the Wernicke-Korsakoff syndrome; (2) the genetic determinants which predispose to the development of thiamine deficiency during the malnutrition associated with chronic exposure to ethanol; (3) the neurochemical and behavioral characterization of an animal model of thiamine deficiency which neuropathologically resembles the Wernicke-Korsakoff syndrome; (4) a preclinical trial of neuropharmacologic agents to reverse the acute thiamine deficiency state (analogous to Wernicke's encephalopathy) and to treat chronic neurochemical and behavioral abnormalities in thiamine-repleted animals (analogous to Korsakoff's psychosis); and (5) a potential association between thiamine deficiency and the etiology of alcoholism.

Methods Employed:

Five each of ten NIH inbred rat strains received a thiamine-deficient (TD) diet (Bioserv Inc., Frenchtown, NJ) in order to evaluate their relative sensitivities to thiamine deficiency. The duration of time required for all animals in a given strain to develop severe neurological signs resembling Wernicke's encephalopathy (ataxia, loss of righting reflex, and convulsions) and subsequently to die was determined. Two strains (M520 and F334) with the smallest intrastrain and the greatest interstrain differences in sensitivities to TD were selected for further study. Animals of both strains were administered either TD or control diet for 2, 5, and 7 weeks, at which times randomly selected animals of each group were given i.p. ethanol 3g/kg. Intoxication, body temperature, and blood ethanol concentrations (BEC) were determined every 90 minutes for 10.5 hours. Two days after ethanol administration, the rats were sacrificed and liver alcohol dehydrogenase (ADH) and catalase were measured, using accepted assay methods (Buhler and Wartburg; Bauduin, respectively). Individually housed rats of both strains were allowed access to only 10% (v/v) ethanol/water solution for 3 days.

Thereafter, they were given a free choice of either water or the 10% ethanol solution for 28 days. Ethanol, water, and food consumption were recorded and the ethanol preference ratio (volume ethanol solution/volume water) was calculated daily. Rats were weighed weekly. The rats were allowed access to only water for 2 weeks; thereafter, the free choice paradigm was once again initiated and the same parameters as previously were recorded. In order to evaluate the role of TD in the behavioral teratogenic potential of prenatal ethanol exposure, we administered the competitive thiamine antagonist pyrithiamine to pregnant Sprague-Dawley rats while they were receiving either TD or control diet. Postnatally, all rats were housed with surrogate mothers who had never been thiamine-deficient, and they received control diet upon weaning. At 6 months of age, the animals were tested in a holeboard apparatus on two occasions separated by 48 hours to investigate whether differences in exploratory activity or its habituation existed between groups. The animals were also tested for their abilities to acquire a passive avoidance task. Thereafter, males were placed in an airtight chamber in which the ethanol concentration in inhaled air could be regulated. BEC and behavioral ratings were evaluated at baseline and after 12 hours (inspired air ethanol concentration 25 mg/L), 24 hours (inspired air ethanol concentration 38 mg/L), and at 3 hours and 6 hours after discontinuation of ethanol.

Major Findings:

The TD-sensitive strain, M520, consistently metabolized ethanol more rapidly and was less intoxicated at an equivalent BEC than the TD-resistant strain, F344. Liver ADH activity was not significantly different in the two strains, but catalase activity was consistently greater in M520 rats. Furthermore, although neither strain was malnourished or lost weight during the free-choice (ethanol solution or water) paradigm, M520 rats preferred the 10% ethanol solution and consumed an average of 10-15 times more ethanol than the F344 rats. After 7 weeks of TD diet, M520 rats showed a greater reduction of baseline body temperature (suggesting hypothalamic damage) and of peak BEC (reflecting either increased volume of distribution or metabolism) than F344 rats compared to their respective controls, confirming their increased sensitivity to TD. Preliminary observations suggest that both strains showed approximately twofold increases in liver ADH activity during TD, but no significant change in liver catalase activity. Rats that had been TD *in utero* had smaller birth weights compared to controls and showed significant growth retardation analogous to that observed in fetal alcohol syndrome. No significant differences in directed exploration (head-dipping) were found between the groups on either day. Furthermore, the groups did not differ significantly in their locomotor activities on the first test day. However, on the second test day, both male and female offspring of thiamine-deficient females had locomotor activities significantly above those of their pair-fed controls. No significant differences between groups were observed in the passive avoidance test.

Significance to Biomedical Research and the Program of the Institute:

Our previous study has demonstrated that the pharmacologic response to ethanol as measured by a behavioral intoxication scale and hypothermia is significantly diminished in rats several months after a severe bout of thiamine deficiency at a time when they appear to have fully recovered their physical health. This suggests that organ damage due to thiamine deficiency may contribute to the

pharmacodynamic and pharmacokinetic tolerance to ethanol observed after chronic exposure to ethanol. Possible explanations for this finding are alterations in central nervous system (CNS) sensitivity to ethanol and/or in the disposition of ethanol. In both male and female rats, past thiamine deficiency reduced the area under the BEC curves by approximately 30%. Theoretically, this could be accounted for by a combination of decreased bioavailability, increased volume of distribution, or increased clearance of ethanol in TD-recovered animals. Preliminary findings of increased liver ADH activity in acutely thiamine-deficient rats are consistent with a change in ethanol metabolism during thiamine deficiency. Whether this is related to changes in turnover of ADH or to induction of a more active isoenzyme needs to be determined. Nonspecific effects of stress and malnutrition that are concomitants of thiamine deficiency need to be excluded. Preliminary findings suggest that a similar phenomenon may be operative in animals who are made thiamine-deficient in utero by using pyrithiamine. The role of TD in the effects of prenatal ethanol exposure on the fetus needs to be further explored.

Perhaps the major significance of these findings is that in our animal model, as in the human condition, there is a broad variability in susceptibility to thiamine deficiency. Understanding the determinants (genetic or otherwise) of this variability may provide insights into alcoholism and human malnutrition, particularly thiamine-deficiency states. In our society, alcoholism is the most common cause of malnutrition and there is much evidence that malnutrition, particularly thiamine deficiency, may play an important role in several of the complications of alcoholism. The fact that the inbred rat strain with greater TD sensitivity prefers ethanol solution and consumes larger amounts of ethanol than the TD-resistant strain needs to be further evaluated. Preliminary findings show that the TD-sensitive rats have reduced CNS sensitivity to ethanol and increased metabolism of ethanol not explained by differences in liver ADH but associated with increased liver catalase activity.

Proposed Course:

We plan to study alcohol dependence and withdrawal as well as learning in previously recovered TD animals. We have also begun long-term experiments examining the effects of repeated thiamine deficiency on these variables. TD-resistant and TD-sensitive strains will be further evaluated for their relative susceptibility to the complications of ethanol exposure, including learning deficits, fetal alcohol syndrome, and appetite for alcohol.

Publications:

Martin, P.R., Majchrowicz, E., Marietta, C., Mukherjee, A.B., and Eckardt, M.J.: Response to ethanol reduced by past thiamine deficiency. Science 227:1365-1368, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00231-03 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Central and Peripheral Nervous System Function in Abstinent Alcoholics

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

PI:	P. Martin	Visiting Scientist	LCS, NIAAA
Others:	M. Eckardt	Section Chief	LCS, NIAAA
	R. Eskay	Research Physiologist	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*

Human Genetics Br., NICHD (A. Mukherjee); Lab. of Psychol. & Psychobiol., NIMH (H. Weingartner); Clin. Psychobiol. Br., NIMH (W. Mendelson, L. Tamarkin); Lab. of Neurorad. & Comp. Tomo., NINCDS (R. Brooks); Nuclear Medicine, CC (S. Larson).

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Science

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

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 of 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 REM 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.

PROJECT DESCRIPTION:

Investigators:

P. Martin	Visiting Scientist	LCS, NIAAA
B. Adinoff	Medical Staff Fellow	LCS, NIAAA
J. Bisserbe	Visiting Fellow	LCS, NIAAA
G. Bone	Medical Staff Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
R. Eskay	Research Physiologist	LCS, NIAAA
J. Johnson	Research Psychologist	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Rio	Staff Fellow	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
N. Salem	Research Chemist	LCS, NIAAA
R. Brooks	Research Scientist	NRCT, NINCDS
S. Larson	Chief	NM, CC
W. Mendelson	Research Psychiatrist	CPB, NIMH
A. Mukherjee	Section Chief	HGB, NICHD
L. Tamarkin	Research Biologist	CPB, NIMH
H. Weingartner	Research Psychologist	LPP, NIMH

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 illnesses 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 fibroblasts 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 to 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 6 months) and four groups of patients (detoxified alcoholics who have been abstinent from alcohol for at least 1 week; alcoholics withdrawn from alcohol who have been abstinent for at least 3 weeks; alcohol amnestic patients; and alcoholic dementia patients), using the following clinical, physiological, and neurochemical tests: (1) skin biopsy for culture of fibroblasts and measurement of thiamine-requiring enzymes (e.g., transketolase), using an assay developed by Dr. Mukherjee's laboratory; (2) neuropsychological evaluation of patients to determine whether they are predominantly amnestic or demented (in collaboration with Dr. Weingartner); (3) norepinephrine response to orthostasis; (4) dose-response to norepinephrine infusion; (5) norepinephrine and endocrine responses to insulin tolerance test; (6) catecholamine and neuropeptide metabolism in cerebrospinal fluid versus plasma and urine; (7) vasopressin response to hypertonic saline infusion; (8) thyrotropin-releasing hormone and gonadotropin-releasing hormone stimulation test; (9) dexamethasone suppression test; (10) corticotropin-releasing hormone test; (11) circadian rhythms of melatonin, body temperature, and activity (in collaboration with Dr. Tamarkin); (12) sleep EEG (in collaboration with Dr. Mendelson); (13) therapeutic trial of the serotonin uptake blocker fluvoxamine; and (14) positron emission tomography, computerized axial tomography, and nuclear magnetic resonance imaging (in collaboration with Drs. Brooks and Larson).

Major Findings:

Preliminary analysis of the corticotropin-releasing hormone (CRH) test results shows no significant differences between alcoholics and normal controls in either baseline levels or magnitude of the adrenocorticotropin (ACTH) or cortisol responses. No significant differences could be demonstrated among 1-week and 3-week abstinent alcoholics and alcoholics with and without organic brain syndromes.

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 5-10% of chronic alcoholics develop this devastating 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 syndrome if they abuse alcohol. We plan to develop a clinical, physiological, and biochemical classification system of alcoholism-associated chronic organic brain syndromes; this system will have diagnostic, prognostic, and therapeutic applications. Our findings to date have been consistent with an amnestic/demented dichotomous classification system. Our new observation of a

normal corticotropin response to CRH in alcoholic patients was unexpected. It is an important negative finding in light of the hypercortisolemia frequently observed in alcoholism and our own findings of impaired pineal function in alcoholics with chronic organic brain syndromes. A therapeutic trial using the serotonin uptake blocker fluvoxamine is an attempt to further test the validity of our classification system of alcoholic organic brain syndromes and to develop an effective treatment for these heretofore untreatable disorders.

Proposed Course:

Data analyses are beginning for many parts of this project.

Publications:

Martin, P.R., Ebert, M.H., Gordon, E.K., Weingartner, H., and Kopin, I.J.: Catecholamine metabolism during clonidine withdrawal. Psychopharamacology 84: 58-63, 1984.

Martin, P.R., Higa, S., Burns, R.S., Tamarkin, L., Ebert, M.H., and Market, S.P.: Korsakoff's psychosis: Decreased 6-hydroxymelatonin excretion. Neurology 34: 966-968, 1984.

Martin, P.R., Weingartner, H., Gordon, E.K., Burns, R.S., Linnoila, M., Kopin, I.J., and Ebert, M.H.: Reply to McEntee, W.J., Mair, R.G.: Catecholamines and amnesia: The need for psychometric verification. Ann. Neurol. 16:516-517, 1984.

Martin, P.R., and Eckardt, M.J.: Pharmacological interventions in chronic organic brain syndromes associated with alcoholism. In Naranjo, C.A., and Sellers, E.M. (Eds.): Research Advances in New Psychopharmacological Treatments for Alcoholism. Amsterdam, Elsevier (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00249-02 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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:	P. Martin	Visiting Scientist	LCS, NIAAA
Others:	B. Adinoff	Medical Staff Fellow	LCS, NIAAA
	G. Bone	Medical Staff Fellow	LCS, NIAAA
	M. Eckardt	Research Psychologist	LCS, NIAAA
	R. Eskay	Research Physiologist	LCS, NIAAA
	D. George	Medical Staff Fellow	LCS, NIAAA
	E. Lane	Staff Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*

N. Salem	Research Chemist	LCS, NIAAA

None

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Science

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

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 decarboxylase 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:

P. Martin	Visiting Scientist	LCS, NIAAA
B. Adinoff	Medical Staff Fellow	LCS, NIAAA
G. Bone	Medical Staff Fellow	LCS, NIAAA
M. Eckardt	Research Psychologist	LCS, NIAAA
R. Eskay	Research Physiologist	LCS, NIAAA
D. George	Medical Staff Fellow	LCS, NIAAA
E. Lane	Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
N. Salem	Research Chemist	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 consumption.

There is now considerable evidence that a 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.

Methods Employed:

Alcoholic outpatients will 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 will be measured by pill count and determination of blood L-DOPA, L-5-hydroxytryptophan, and carbidopa levels. Alcohol consumption will be 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, and weekly sweat patch tests (which provide integrated information concerning alcohol consumption during the previous week). 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 will allow neurochemical classification of

patients and determination of whether patient and neurochemical characteristics are related to treatment outcome.

Major Findings:

To date, 10 patients have been admitted to the protocol: 1 has successfully completed the 1-year study, 4 are actively participating, and 5 have resumed drinking and 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:

This project is continuing. Initial analysis of the results will be carried out when 10 patients have entered each cell of the study.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00260-01 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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:	P. Martin	Visiting Scientist	LCS, NIAAA
Others:	B. Adinoff	Medical Staff Fellow	LCS, NIAAA
	G. Bone	Medical Staff Fellow	LCS, NIAAA
	R. Eskay	Research Physiologist	LCS, NIAAA
	J. Keranian	Senior Staff Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	N. Salem	Research Chemist	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 20205

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 in up to one-third of all hypertensives. The association between hypertension and alcohol consumption awaits causative explanation and is the purpose of this study. Blood pressure will be measured using a 24-hour ambulatory blood pressure monitoring system for several days in normotensive and hypertensive social drinkers during periods of usual alcohol consumption and abstinence. Subjects will be on a low monoamine diet for the duration of the study. Blood and urine samples will be 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 will be related to blood pressure in the three phases of the study.

PROJECT DESCRIPTION:

Investigators:

P. Martin	Visiting Scientist	LCS, NIAAA
B. Adinoff	Medical Staff Fellow	LCS, NIAAA
G. Bone	Medical Staff Fellow	LCS, NIAAA
R. Eskay	Research Physiologist	LCS, NIAAA
J. Keranian	Senior Staff Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
N. Salem	Research Chemist	LCS, NIAAA
H. Keiser	Chief	HE, NHLBI

Objectives:

The association between alcoholism and hypertension was first reported 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 activities, 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 its 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 1-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 of 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 2 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 a.m. and 4 p.m. On arrival, a double stop-cocked i.v. line will be placed for blood sampling and BP will be monitored by a Dynamapp automatic blood pressure machine. After the subjects rest for 2 hours, blood will be withdrawn for homovanillic acid, norepinephrine, 3-methoxy-4-hydroxyphenylglycol, serotonin, epinephrine, vasopressin, adrenocorticotropin (ACTH), atrial natriuretic factor (ANF), prostacyclin, thromboxane, renin, angiotensin, magnesium, ionized calcium, and creatinine concentrations. Immediately after the blood sample is obtained, subjects will stand and blood pressure will be measured every minute for 5 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 2 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 2 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 2 weeks later. Blood and urine will be studied when blood pressure is being monitored, as in the first part of the study.

Major Findings:

This project has just commenced.

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 address this issue directly.

Proposed Course:

The project has just commenced and will continue for 2 years.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00261-01 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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:	P. Martin	Visiting Scientist	LCS, NIAAA
Others:	B. Adinoff	Medical Staff Fellow	LCS, NIAAA
	G. Bone	Medical Staff Fellow	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	E. Majchrowicz	Research Chemist	LPS, NIAAA
	C. Marietta	Physiologist	LPS, NIAAA
	J. Rohrbaugh	Research Psychologist	LCS, NIAAA

COOPERATING UNITS *(if any)*

F. Weight, Chief, LPS, NIAAA

Walter Reed Army Institute of Research (T. Jerrells); Clinical Psychobiology Branch, NIMH (W. Mendelson, L. Tamarkin).

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Studies

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

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 etiologic mechanism(s) 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 will be performed on acutely withdrawing alcoholics during withdrawal and 3 weeks after withdrawal. Plasma melatonin, cortisol, and ACTH will be determined at 3-hour intervals, and hand tremor will be repeatedly measured. Lying-standing norepinephrine determinations will be made twice during the initial 72 hours and once weekly for 3 weeks. Alcoholic patients and age- and sex-matched controls will be evaluated for general immunocomponents to determine the effects of long-term alcohol abuse and withdrawal from alcohol on the immune system. Patients will be evaluated at regular intervals to investigate effects of varying durations of abstinence from alcohol on selected parameters of immunocompetency.

PROJECT DESCRIPTION:

Investigators:

P. Martin	Visiting Scientist	LCS, NIAAA
B. Adinoff	Medical Staff Fellow	LCS, NIAAA
G. Bone	Medical Staff Fellow	LCS, NIAAA
M. Eckardt	Research Psychologist	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
E. Majchrowicz	Research Chemist	LPS, NIAAA
C. Marietta	Physiologist	LPS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
T. Jerrells	Immunologist	WRAIR
W. Mendelson	Research Psychiatrist	CPB, NIMH
L. Tamarkin	Research Biologist	CPB, NIMH
F. Weight	Chief	LPS, NIAAA

Objectives:

The alcohol withdrawal syndrome is characterized by pathophysiological changes in many organ systems. In this project, we explore etiologic mechanism(s) 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 have a positive breathalyzer reading for ethanol on admission will wear ambulatory EEG electrodes, an activity monitor, and a rectal temperature probe for continuous 24-hour monitoring. An indwelling venous catheter will be inserted in an antecubital vein for blood sampling every 3 hours, around the clock, for determination of melatonin, cortisol, and ACTH. Urine will be collected for determination of catecholamines and metabolites. Monitoring and sampling procedures will be conducted for 72 hours after entry into the protocol. These 72-hour monitoring procedures will be repeated 21 days after admission to the study. Lying and standing blood samples for determination of catecholamines will be obtained 48 hours and 120 hours after the breathalyzer reading reaches zero. This procedure will be repeated once a week with concomitant 24-hour urine collection for catecholamine metabolites. The procedure will be performed a total of five times per patient.

In the present study, we also propose to evaluate general parameters of immunocompetency in alcoholic patients and age- and sex-matched controls. Lymphocytes from peripheral blood will be evaluated for their ability to respond to nonspecific mitogens, recall antigens, and allogeneic tissue antigens (mixed lymphocyte response). Alterations in the humoral immune response will be evaluated by measuring antibody production in culture after stimulation of cells with polyclonal activators such as pokeweed mitogen as well as measuring immunoglobulin levels in sera. Lymphocyte function will also be monitored by measurement of lymphokine production, which is a function of the T-cell. 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 will be 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 will be accomplished using fluorescent-activated cell sorter techniques and will address whether alterations in lymphocyte type induced by ethanol result in the immune alterations previously reported. Plasma obtained from lymphocyte isolation procedures will be saved and stored for evaluation of antibodies reactive against the patients' lymphocytes and other human tissues and cell lines as available. Fluorescent-activated cell sorting techniques will be used to evaluate autoantibody reactivity.

Major Findings:

This project has just commenced.

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 alcoholics following abstinence may be related to REM rebound. We have demonstrated that the 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.

In humans, excessive ethanol consumption has been associated with a number of defects in specific and nonspecific host defense 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 mobilization 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 have some 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 aspects of the alcohol withdrawal syndrome.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00233-03 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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:	Y. Davenport	Research Social Worker	LCS, NIAAA
Others:	M. Linnoila	Chief	LCS, NIAAA
	P. Martin	Visiting Scientist	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	H. Moss	Medical Staff Fellow	LCS, NIAAA
	V. Moore	Research Social Worker	LCS, NIAAA
	J. Johnson	Research Psychologist	LCS, NIAAA

COOPERATING UNITS *(if any)*
Social Work Department, Clinical Center, NIH (D. Rooney); Division of Educational Research Programs, Behavioral Science Research Branch, NIMH (A. King).

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Science, Unit of Family Studies

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
3.0	3.0	

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) Minors

☒ (a2) Interviews

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

The recruitment and assessment of families in which a diagnosis of primary alcoholism can be made for the identified patient remains a major effort of the Unit and provides us with a population of diagnostically appropriate research subjects for study. A particular focus this year has been the selection and evaluation of normal control subjects and their families able to participate in pharmacological as well as psychosocial studies conducted on the Research Ward and in the Outpatient Clinic. Studies of experiential factors in the occurrence of familial alcoholism from a multigenerational perspective, the identification of persons at risk, and studies of efficacious methods of intervention remain primary research objectives. In an ongoing homogeneous couples psychotherapy group, we are evaluating this modality as a cost-effective method for the treatment of both the alcoholism and marital and family conflicts of the married alcoholic patient. An adult children of alcoholics psychotherapy group is in place to assess the adaptive mechanisms of nonalcoholic offspring in order to compare these and other variables found in their alcoholic siblings. The fact that other psychiatric disorders coexist with alcoholism is known. Another ongoing study concerns the relationships of the appearance of affective disorders and alcoholism in a single large family pedigree. Affective disorders are of particular interest since, like alcoholism, they show evidence of genetic heritability and the existence of biological components. Data collection will continue until we have a sample of 200 alcoholic patients and their families and 50 normal control families.

PROJECT DESCRIPTION:

Investigators:

Y. Davenport	Research Social Worker	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
P. Martin	Visiting Scientist	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
H. Moss	Medical Staff Fellow	LCS, NIAAA
V. Moore	Research Social Worker	LCS, NIAAA
J. Johnson	Research Psychologist	LCS, NIAAA
E. Mathiasen	Social Science Analyst	LCS, NIAAA
B. Orlans	Guest Researcher	LCS, NIAAA
D. Rooney	Social Worker	CC, NIH
A. King	Social Worker	BSRB, NIMH

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 patients (women, blacks, and Hispanics) who are alcohol abusers; (3) longitudinal studies of children at risk, focusing on predictive factors and early detection; and (4) 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, Family Environment Scale (Moos), and Locke-Wallace Marital Adjustment Scale, as well as other instruments which will enable us to quantitate biopsychosocial characteristics.

Major Findings:

We are in the process of coding families and assembling a data base. In the population we are studying, our preliminary observations are the following: (1) an extensive amount of affective disorders, dysthymia, and anxiety among family members of alcoholics; (2) an extensive amount of secondary depression among probands; (3) a sporadic appearance of anorexia and bulimia in female patients and female relatives; (4) a considerable amount of violence and sociopathy in the families; and (5) evidence of assortative mating. We are documenting the toll alcoholism has taken on many families through generations. Most of the characteristics we have observed have been noted by previous investigators to be associated with the presence of alcoholism, but their interrelatedness is still not well understood.

Research findings from the married couples group related to group evolution and group process were presented in April at the annual meeting of the American Orthopsychiatric Association. The initial preoccupation was with concerns related to

(1) genetics and heritability, (2) abusive behavior, (3) impairment of occupational and social skills, and (4) problems related to uncontrolled drinking. The group focus is now concentrated on (1) improved communication between spouses, (2) role reversal issues, (3) restoration of function, (4) painful effects from the past which were previously denied, and (5) changes in drinking behavior. These changes were reflected in scales used to measure marital and social adjustment, in weekly self-administered forms, tape-recorded sessions, and therapists' observations. The cost-effective advantages of the paradigm are that (1) more people can be treated with fewer staff members and (2) more families can be reached by concentrating on the marital dyad in a group modality. An article reporting these and other results is near completion.

Significance to Biomedical Research and the Program of the Institute:

Because the consequences of abusive drinking affect not only the individual patient but the family as well, it is important to examine the significance of biopsychosocial influences in the development and treatment of alcoholism. This can best be done through longitudinal studies of alcoholic patients and their families, which we are undertaking.

Proposed Course:

The long-term course will focus on better understanding the interplay between biogenetic factors and environmental events, especially dysfunctional patterns of family interaction. The addition of a full-time social worker in January 1984, 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 data will determine additional research directions.

Publications:

Davenport, Y., and Adland, M.: Issues in the treatment of the married bipolar patient: denial and dependency. In Journal of the American Psychiatric Association (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00234-03 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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	Unit Chief	LCS, NIAAA
Others:	R. Cotton	Staff Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	P. Rathnagiri	Visiting Fellow	LCS, NIAAA
	K. Valkonen	Visiting Fellow	LCS, NIAAA
	R. Lister	Visiting Fellow	LCS, NIAAA
	J. Johnson	Research Psychologist	LCS, NIAAA

COOPERATING UNITS (if any)
Biological Psychiatry Branch, NIMH (L. Goldin and C. Merril); Laboratory of Viral Carcinogenesis, NCI (S. O'Brien); VA Medical Center, Portland, OR (J. Crabbe).

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Unit of Genetic Studies

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
10.0	6.0	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.)

To identify unknown genetic loci determining alcoholism, we are testing for linkage or association between genetic markers and behavioral phenotypes. The markers are protein polymorphisms, DNA restriction fragment polymorphisms (RFLP's), and classical allozymes and blood antigens. The probability of establishing linkage or association is being maximized by (1) focusing on human alcoholism with impulsivity/aggressivity as a prominent accompanying trait, (2) utilizing mouse genetic models, and (3) using a large panel of polymorphisms. Markers include RFLP's defined by probes specific for the Y chromosome. These are being tested, using DNA from cell lines established from male alcoholics. In addition, we mapped 40 protein polymorphisms by two-dimensional electrophoresis of the abundant proteins of lymphocytes, fibroblasts, erythrocytes, and serum. We showed two of the unknown loci to be phosphoglucomutase-3 and glyoxalase-1. Our population studies using this technique revealed that the overall level of genetic variability, as indexed by the average heterozygosity of cellular proteins, is between 2% and 2.5%, one-third the level previously accepted for the human. In the mouse, we have identified 12 brain polypeptide variants and have successfully correlated 1 of these with resistance to ethanol-withdrawal seizures. We have also shown that the short- and long-sleep mouse strains, widely used to investigate determinants of sensitivity to acute ethanol-induced sedation, are highly inbred, decreasing their usefulness in correlational studies. We are studying the known enzymes of ethanol metabolism, principally the alcohol and aldehyde dehydrogenases, at the protein and DNA levels. In this work, we have recently developed a new technique for the electrophoretic characterization of variants of these proteins: immobiline isoelectric focusing. At the DNA level, we have completed a linkage analysis of an alcohol dehydrogenase RFLP to chromosome 4 markers as well as a preliminary survey of the alcohol dehydrogenase genes present in the hominoid primates.

PROJECT DESCRIPTION:

Investigators:

D. Goldman	Unit Chief	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
R. Cotton	Staff Fellow	LCS, NIAAA
P. Rathnagiri	Visiting Fellow	LCS, NIAAA
K. Valkonen	Visiting Fellow	LCS, NIAAA
R. Lister	Visiting Fellow	LCS, NIAAA
J. Johnson	Research Psychologist	LCS, NIAAA
C. Merril	Surgeon	BPB, NIMH
L. Goldin	Staff Fellow	BPB, NIMH
S. O'Brien	Senior Staff Fellow	LVC, NCI
J. Crabbe	Staff Scientist	VA Research, Portland, OR

Objectives:

The approach to these studies was determined in recognition of the fact that alcoholism arises from multiple genetic and environmental factors that can usually be observed only in combination. Strategically, it is possible to better isolate and define particular components in family studies; in families, the environmental and genetic heterogeneities are less and individual components are placed in relief. In addition, inbred animal and cellular models can be used to study particular components in depth. The new molecular genetic methods also offer the possibility of providing markers to make some basic genetic determinants accessible.

To maximize the extent of genetic homogeneity, emphasis is being directed toward studying families and individual males that show alcoholism associated with impulsivity/aggression. This is a particular subtype of alcoholism, which we have chosen as the initial focus of study for several reasons: (1) studies done elsewhere indicate that alcoholism associated with impulsivity/aggression is genetically transmitted and (2) the impulsive/aggressive phenotype is, in both humans and animals, associated with lower central serotonergic activity and the finding of extra or abnormally long Y chromosomes in males. This provides a possible connection to the observed greater prevalence of alcoholism in males (3-5%) than in females (0.1-1%). Also, families manifesting autosomal forms of genetic transmission are being developed for study by the Unit on Family Studies within the LCS. When available, large pedigrees showing autosomal genetic transmission of a reliable phenotype associated with alcoholism will be studied, using protein and DNA linkage markers.

The objectives of these studies are (1) to use protein polymorphisms detected by two-dimensional protein electrophoresis and by other methods to establish molecular markers and locate genes responsible for alcoholism-associated behavioral differences; (2) to chromosomally map recently discovered polymorphic loci and to develop additional polymorphisms to improve the probability of establishing genetic linkage; (3) to study, in humans and other animals, the natural variants and evolution of enzymes of ethanol metabolism, principally alcohol and aldehyde dehydrogenases, at the protein and DNA levels; (4) to utilize specific DNA

probes for the Y chromosome to study male alcoholism associated with impulsivity; and (5) to isolate and study novel genetic differences relevant to alcohol consumption in humans and mice.

Methods Employed:

Clinical studies of familial alcoholics and their relatives are being accomplished in collaboration with the Unit on Family Studies (LCS, NIAAA), Dr. J. Johnson (LCS, NIAAA), and Dr. L. Goldin (NIMH). Each family member receives a diagnostic interview and completes the MMPI (which yields subscale data normative for impulsivity). Some individuals with other manifestations of the impulsive/aggressive trait will be studied. These include violent prisoners and individuals with histories of suicidal behavior. The family members studied by Dr. Johnson will undergo a variety of neuropsychological tests.

For protein and DNA genetic studies, a blood sample and a skin biopsy are obtained. Skin biopsies are used to establish primary skin fibroblast cultures. Sufficient cells are cultivated to provide material for analysis of allozyme polymorphisms, protein polymorphisms by two-dimensional electrophoresis, DNA for analysis with Y chromosome and specific gene probes. Cells preserved in liquid nitrogen can later be grown for additional studies. Human liver specimens for studying genetic variants of enzymes of ethanol metabolism will be obtained from surgical biopsy specimens.

For protein-level studies of alcohol and aldehyde dehydrogenases, we have recently applied a new technique, immobiline isoelectric focusing, for the separation and purification of these enzymes. In immobiline isoelectric focusing, a charge gradient is fixed at the time the gel is cast so that proteins can be focused at voltages as high as 5000 V to give improved separations.

Polymorphisms are genetic variants with an allelic frequency of greater than 1% in the normal population, which may be used as markers in genetic linkage studies. Some variants, such as the known Oriental variants of alcohol and aldehyde dehydrogenases, are genetic determinants of human phenotypic differences. Protein polymorphisms by two-dimensional electrophoresis are generated by subjecting proteins of erythrocytes, serum, cultured fibroblasts, and cultured lymphoblasts to separation by isoelectric point in the first stage and separation on the basis of size in the second stage. Polypeptides are visualized by ultrasensitive silver stains developed by Dr. Merril (LGCB, NIMH) and demonstrated to be suitable for quantitative studies by Dr. Merril and Dr. Goldman, or by autoradiography of isotopically labeled proteins. Additionally, allozyme and serological polymorphic phenotypes are determined.

Polymorphisms of primary DNA sequence manifest themselves because they alter the sites where the restriction enzyme(s) cuts the DNA and therefore alter the sizes of the resulting DNA fragments. These restriction fragment-length polymorphisms are detected by isolating DNA and digesting it to completion with the restriction enzyme of interest. The resulting DNA fragments are separated according to size by agarose gel electrophoresis. DNA in the gel is denatured and transferred to a nitrocellulose filter, resulting in a replica of the pattern of fragments in the gel. To visualize the RFLP's, a cloned DNA sequence is labeled with ^{32}P and hybridized to the DNA on the filter, resulting in the detection of specific

fragments. In this fashion, we have very recently utilized the MSP-1 polymorphism of alpha-ADH in a genetic linkage study with the chromosome 4 loci Gc and MNS.

In collaboration with Dr. L. Goldin (NIMH), a computer program (LIPED) is used to test for genetic linkage between protein and DNA sequence polymorphic loci and other genetic loci. The linkage method depends on the demonstration of nonrandom genetic assortment of two genetically determined traits. This occurs when the two traits have chromosomal loci which lie close enough to one another that they will not be separated as often by recombination. We are also using the linkage approach to localize newly described polymorphic loci.

For genetic linkage studies, the fraction of the genome which can be analyzed is largely determined by the number of polymorphic markers available. This is because a polymorphic marker locus must lie relatively close to a locus for this physical association to be proven by demonstrating nonrandom assortment in families. We have constructed a large panel of protein polymorphisms (approximately 50) by supplementing the conventionally detected protein polymorphisms with 40 polymorphisms that we can detect on two-dimensional electrophoretograms of serum, erythrocytes, lymphocytes, and cultured fibroblasts. This increases the fraction of the human genome covered at a linkage distance of 10 centimorgan from 14% to more than 25%.

The distribution of alcohol and aldehyde dehydrogenases in rodent and primate brain is being studied, using immunohistochemical staining and enzyme assays. Enzyme activity measurements are made spectrophotometrically. For immunohistochemical studies, polyclonal antibodies against alcohol dehydrogenases have been prepared by immunizing rabbits, and monoclonal antibodies have been prepared by immunizing Balb/C mice, fusing their splenocytes with myeloma cells, and selecting positive clones by means of an ELISA assay. These antibodies are allowed to incubate with fixed, perfused tissue sections. The sections are washed extensively, and specific antibody binding sites are stained using a sensitive biotin-avidin technique.

For the studies using mice, we have identified 12 polymorphic mouse brain protein variants by two-dimensional electrophoresis. To identify variant loci determining alcoholism-associated genetic differences, we have used these brain protein variants to screen mouse strains differing in sleep time after ethanol administration (short-sleep and long-sleep) and mouse strains differing in susceptibility to ethanol-withdrawal symptoms. These strains have been bred specifically to minimize inbreeding while the trait is selected, so that any fixed genetic differences should relate to the trait for which the selection was conducted. In the study on determinants of ethanol-withdrawal severity, we had available to us (from Dr. J. Crabbe, VA Research, Portland, OR) replicate lines of withdrawal seizure-resistant, -sensitive, and control mice. It is therefore highly probable that a genetic variant we have found to be fixed in both replicate withdrawal seizure-resistant strains is an actual determinant or genetic marker. Confirmation of this finding is being attempted by screening a large number of mice that are heterogeneous at this locus. Additional mapping and behavioral correlation studies on inbred and recombinant inbred mouse strains are being done in collaboration with Dr. R. Lister of the Section on Neurochemistry (LCS).

For quantitative analysis of polymorphic proteins or DNA fragments, we have implemented a computerized image analysis system which enables an operator to rapidly make measurements of relative densities and positions in a semiautomated fashion.

For evolutionary studies, skin biopsies and cell lines from a variety of primates and other animals have been obtained from Dr. S. O'Brien (NCI) and other sources. Primary fibroblast cell lines are established from skin biopsies, and DNA is purified from these sources. DNA is cut with restriction enzymes and analyzed with DNA probes specific for alcohol and aldehyde dehydrogenases (provided by Dr. A. Yoshida, City of Hope, and Drs. G. Duester and M. Smith, UC Irvine). Primate alcohol and aldehyde dehydrogenase sequences will be isolated from primate genomic libraries recently made available to us by Dr. J. Slightom (Upjohn). The primate libraries will be screened, using subcloned portions of the human alcohol and aldehyde dehydrogenase DNA probes. Primate sequences will be determined and can then be compared with human sequences and with each other, using comparison programs made available by J. Felsenstein and implemented on our VAX computer. DNA regions of particular interest to us include the 5' promoter and regulatory regions and the coding sequences.

Major Findings:

Human protein polymorphisms by two-dimensional electrophoresis. In population and family studies, 40 protein polymorphisms have now been identified by two-dimensional electrophoresis of human lymphocytes, fibroblasts, serum, and erythrocytes. Approximately half of these were first identified by our group. In population and family studies of human serum, erythrocyte and fibroblast proteins, we have detected 7, 4, and 17 polymorphic loci, respectively, on two-dimensional electrophoretograms and have verified that most are transmitted in Mendelian codominant fashion. We have shown that 6/16 of these polymorphic loci detectable in the fibroblast are also detectable in the lymphocyte. We have recently established the identity of one of these polymorphic loci detected in fibroblasts as phosphoglucomutase 1. A second locus, detected in erythrocytes, is glyoxalase 1. Using the family studies approach, we have also shown linkage between the unknown fibroblast locus NC22 and apolipoprotein E (Lod score = 2.8 at 0 recombination) and a possible linkage between the unknown serum locus Ser1 and alpha haptoglobin.

Human population genetic variability. These studies have established a minimum level of average cellular protein heterozygosity (genetic variability) of approximately 2.2% for the most predominant human cellular proteins (from fibroblasts and lymphocytes) and 5.6% for the most predominant proteins of serum. In contrast, two animals that show less genetic variability, the cheetah (with an average heterozygosity of 1.3%) and the panda, showed less genetic variation in our studies. Our studies have called into question earlier estimates of very low human genetic variability (in which average heterozygosity was stated to be less than 1%), obtained by using two-dimensional electrophoresis. Our estimates are approximately one-third the rates estimated on the basis of allozyme heterozygosity.

Mouse models for alcoholism-associated behaviors. In the area of mouse genetic models for alcoholism-associated behaviors, we (with Drs. R. Deitrich and R. Baker, Denver, CO) have demonstrated 12 genetic variant protein loci in mouse brain by two-dimensional electrophoresis. We (with Dr. J. Crabbe, VA Research, Portland, OR) have shown that one of these, an abundant 71-kilodalton, 5.4-isoelectric point protein may be a determinant of severity of ethanol withdrawal. This finding was made by studying the variant brain loci in two strains of mice bred for severe withdrawal, two strains selected for mild withdrawal, and two nonselected control strains. All of the strains were derived from a common, genetically heterogeneous stock and have been maintained in a highly outbred state. Therefore, our finding that only the two strains selected for mild withdrawal are genetically fixed at this locus while the two strains selected for severe withdrawal show a gene frequency change in the opposite direction suggests this locus is a determinant of withdrawal severity. We are currently attempting to confirm this association.

In a second mouse study (with Drs. Deitrich and Baker), we showed that the short-sleep and long-sleep mice, two strains widely used to investigate determinants of sensitivity to the acute sedative effects of ethanol, are highly inbred. This finding is important because it means that correlations found using the long-sleep and short-sleep mouse strains are weakened by the high degree of random genetic fixation in these strains.

Human familial alcoholism and male alcoholism associated with impulsivity. Thirty-five adult male alcoholics have received psychological testing for impulsivity, have had cell lines established in tissue culture, and are being screened by Dr. R. Cotton, using restriction fragment-length polymorphisms specific for the Y chromosome. Dr. Cotton is also isolating additional Y-specific DNA probes from a library of Y-chromosomal sequences obtained from the Lawrence Livermore National Laboratory.

We have recently initiated the establishment of permanent lymphoblastoid lines on alcoholism families undergoing intensive neuropsychological evaluation by Dr. J. Johnson. This will enable genetic mapping and correlative studies to be performed if suitable genetic phenotypic markers for alcoholism are discovered by Dr. Johnson.

Human alcohol and aldehyde dehydrogenases. Dr. Rathnagiri has implemented many of the conventional methods for analysis and purification of alcohol and aldehyde dehydrogenases, including spectrophotometric enzyme measurements, starch gel electrophoresis, and protein purification, using anion exchange and affinity methods. He has produced monoclonal antibodies against a mixture of class 1 alcohol dehydrogenases and is attempting to construct separate panels of monoclonals against each type.

Dr. Valkonen has applied a new method, immobiline isoelectric focusing, to the analytical and preparative separation of alcohol and aldehyde dehydrogenases. She has determined the isoelectric points of these proteins more accurately than could be done previously. She has shown that this method can be used as a rapid preparative procedure so that these enzymes can be isolated from individual liver specimens to be examined for genetic variation in Km and thermal stability.

Evolutionary studies. In the area of evolutionary studies on alcohol dehydrogenases, we have very recently completed an initial screening of DNA's from the great apes and the macaque monkey and have determined that, as in the human, multiple class 1 dehydrogenases are found in all of these species. Isolation of the 5' ends of these alcohol dehydrogenase genes and aldehyde dehydrogenases from primate genomic libraries is in progress. This will be followed by their comparative sequencing.

In a somewhat parallel effort, we have developed a new method for determining genetic distances and phylogenetic relationships between species. In this method, several hundred proteins are compared between species for protein charge variation manifested on two-dimensional gels. In a recently submitted report, we showed that the phylogenetic trichotomy of human, chimpanzee, and gorilla should be broken in favor of the gorilla having diverged prior to the separation of the other two lineages.

Significance to Biomedical Research and the Program of the Institute:

Genetic studies in humans and in animals provide a paradigm for isolating biological factors so that they can be studied with powerful new molecular genetic methods. This type of approach can result in the discovery of genetic factors that participate in determining individual susceptibility to alcoholism.

The brain genetic studies in ethanol-withdrawal-sensitive and ethanol-withdrawal-resistant mice have raised the possibility that we have detected the first brain molecular determinant of ethanol response: a polypeptide whose genetic variants affect sensitivity to ethanol withdrawal. If this genetic correlation can be confirmed, variants at this locus can then be searched for in human or primate brains.

The brain genetic studies in long-sleep and short-sleep mice have uncovered very high levels of inbreeding in these strains, which are widely used by investigators attempting to understand the basis of differences in response to the acute sedative effects of alcohol. These high amounts of inbreeding dictate caution in drawing correlative conclusions with these particular strains.

Our mapping of new human polymorphic loci contributes to the overall process of human gene mapping and provides a resource of linkage markers which can be applied to the future mapping of genetic determinants of human alcoholism. Our studies on human population genetic variability help to better characterize humans as a species.

The introduction of preparative and analytical immobiline isoelectric focusing for alcohol and aldehyde dehydrogenases should facilitate the screening for additional human genetic variants of these proteins. Isolation of novel variants of these proteins would shed additional light on the structure and function of these enzymes which carry out the bulk of ethanol metabolism in the human. The purification and production of monoclonal antibodies to alcohol and aldehyde dehydrogenases will provide improved tools for their analytical detection or cloning.

Theodosius Dobzhansky remarked that "Nothing in biology makes sense except in the light of evolution," meaning that the ways that each organism solves the problems of its existence are largely determined by the history of how that organism and its component parts arose. Our recently completed survey of the class 1 alcohol dehydrogenase genes in the species nearest to the human, and our recently begun efforts to clone and sequence the alcohol and aldehyde dehydrogenases in these animals, will help to make sense of the structure and function of the loci primarily controlling ethanol metabolism in man. Our phylogenetic work in which we have measured the molecular distances between the great apes in a new way, by two-dimensional electrophoresis, strengthens our general understanding of their phylogenetic relationships. This improves our ability to make specific inferences about the events at individual genetic loci, such as the alcohol and aldehyde dehydrogenases.

The establishment of permanent cell lines for complete, intensively studied families with alcoholism and individual male alcoholics creates a resource that will encourage future genetic and in vitro metabolic studies on alcoholism.

The computerized image-analysis methods and genetic biochemical methods developed for these studies provide a technical capability for the Institute and a resource for investigators working in a variety of other areas.

Proposed Course:

For human studies, collection into cell culture of intensively studied families with alcoholism and of male alcoholics typed for impulsivity will continue. In the future, suitable families will be studied, using the linkage method with DNA and protein molecular markers. We will continue to test individual male alcoholics with restriction fragment-length polymorphisms specific for the Y chromosome. Continued effort will be placed on identifying and chromosomally mapping new polymorphic loci discovered by the two-dimensional electrophoresis method for use as linkage markers.

We will attempt to isolate and study new genetic variants of alcohol and aldehyde dehydrogenases from human liver, using analytic and micropreparative methods we have recently developed.

For evolutionary studies on the alcohol and aldehyde dehydrogenase loci, we will clone and sequence the alcohol and aldehyde dehydrogenases in other animals, including the hominoid apes, in order to make better sense of the structure and function of these loci. Special attention will be focused on the 5' regulatory sequences and the coding sequences. Phylogenetic work, in which we measure the molecular distances between these species and the rates of evolutionary change in different regions of these genes, will be performed in parallel.

We will complete the purification of the human alcohol and aldehyde dehydrogenases and the construction of panels of monoclonal antibodies, and may elect to use these as tools for the cloning of the pi or chi alcohol dehydrogenases.

In mouse brain genetic studies, we will attempt to confirm the correlation we have discovered between a genetic variant brain protein and ethanol-withdrawal severity. If it can be proven, we will search for analogous variants at this locus in human brain.

Publications:

Goldman, D., Goldin, L.R., Rathnagiri, P., O'Brien, S.J., Egeland, J., and Merril, C.R.: Twenty-seven protein polymorphisms by two-dimensional electrophoresis of serum, erythrocytes and fibroblasts in two pedigrees. Am. J. Hum. Genet. (in press).

Goldman, D., and Merril, C.R.: Genetic polypeptide variation by two-dimensional electrophoresis. Ann. N.Y. Acad. Sci. 428:186-200, 1984.

Goldman, D., Nelson, R., Deitrich, R.A., Baker, R.C., Spuhler, K., Markley, H., Ebert, M., and Merril, C.R.: Genetic brain polypeptide variants in inbred mouse strains and strains selectively bred for high and low sensitivity to alcohol. Brain Research (in press).

Harrington, M.G., Merril, C.R., Goldman, D., Xu, X., and McFarlin, D.E.: Two dimensional electrophoresis of cerebrospinal fluid proteins in multiple sclerosis and various neurological diseases. Electrophoresis 5:236-245, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00239-02 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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:	P. Martin	Visiting Scientist	LCS, NIAAA
	R. Rawlings	Mathematical Statistician	DBE, NIAAA

COOPERATING UNITS *(if any)*

United States Soldiers' and Airmen's Home, Washington, DC (N. Keller, A. Law, G. Smith); Laboratory of Psychology and Psychopathology, NIMH (H. Weingartner).

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Brain Research

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.5	0.5	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 purpose of this study is to examine longitudinally 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 an alcoholism treatment program; and nonalcoholic controls who are undergoing a routine physical examination. The risk of developing either type of chronic organic brain syndrome with continued alcohol abuse by alcoholics in a treatment program will be determined, as well as the consequences of long-term abstinence or continued alcohol consumption, albeit at significantly reduced levels.

PROJECT DESCRIPTION:

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
P. Martin	Visiting Scientist	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	Unit Chief	LPP, NIMH

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 syndrome. Less cognitively impaired alcoholics will be evaluated similarly and then followed over time to determine whether (1) continued alcohol abuse results in a specific and predictable chronic organic brain syndrome, (2) abstinence results in improved cognitive functioning, or (3) continued alcohol consumption, albeit at reduced levels, results in an adverse effect on cognitive functioning.

Methods Employed:

Two clinically defined groups of alcoholics will be evaluated: (1) participants in an alcoholism treatment program, and (2) alcoholics 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 alcoholics 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. The neuropsychological scores for each alcoholic in the treatment program will then be compared to those with alcoholic dementia and those with alcoholic amnestic syndrome to determine to which group he is most similar. This designation will constitute the predicted outcome variable if the individual continues to abuse alcohol.

It is well established that abstinence can result in improved neuropsychological performance in mildly impaired alcoholics. Moreover, it has been suggested that

continued alcohol consumption, albeit at significantly reduced levels, can reduce this rate of improvement. Some of the cognitive impairments in alcoholic dementia may also improve with abstinence, although the memory dysfunction in alcoholic amnestic syndrome is characterized as severe and persistent. The consequences of abstinence or reduced alcohol consumption will be determined for each of the three alcoholic groups by conducting annual neuropsychological testing. The nonalcoholics will also be subjected to repeated testing to control for any practice effects and for aging.

Initially, each subject will participate in four sessions on 4 separate days. The first three sessions will involve a detailed neuropsychological assessment of intelligence, memory, and other cognitive functions. The fourth session will involve the collection of socioeconomic information, personality assessment, childhood history of hyperactivity, and drugs use history, including alcohol use. At the end of the fourth session, each subject will be asked to provide the names of four collateral sources, friends or relatives, who can be contacted every 6 months to verify the patient's drinking and drinking-related behavior during the previous time period. Patients will be contacted monthly, interviewed in depth every 6 months, and subjected to annual neuropsychological testing for 5 years.

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; Luria-Nebraska Battery; Wechsler Adult Intelligence Scale; Wechsler Memory Scale; Wisconsin Card Sorting Test; Paced Auditory Serial Addition Test; and a selected memory test 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 not yet begun to analyze them.

Significance to Biomedical Research and the Program of the Institute:

It has been well documented that alcoholics have impaired brain function. The course of this impairment with continued alcohol consumption or abstinence has not been well studied; to do so is one of the goals of the present research. Relatively little is also known about the etiology and reversibility of alcoholism-associated dementia, which is the second most common form of dementia. Relationships between alcoholism-associated dementia and amnestic syndromes are unclear and are of importance in planning appropriate pharmacological intervention.

Proposed Course:

Data collection will be continued, with anticipated preliminary results next year.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00240-06 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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
Other:	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 20205

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.)*

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 nonlinear 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 prediction variables, including neuropsychological performance during treatment and subsequent outcome, are currently being analyzed. An initial finding was that an 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.

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, to discern possible etiological factors related to this impairment, and to determine whether improvement in function is associated with subsequent abstinence. Additional questions concern the commencement of treatment immediately after detoxification, the relationships between CNS function and treatment outcome, and the neuropsychological consequences of posttreatment 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 posttreatment 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 males 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 alcoholics who abstain 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.

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.

Proposed Course:

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

Publications:

Eckardt, M.J., and Martin, P.R.: Clinical assessment of cognition in alcoholism. Clin. Exp. Res. (in press).

Eckardt, M.J., Rawlings, R.R., Ryback, R.S., Martin, P.R., and Gottschalk, L.A.: Effects of abstinence on the ability of clinical laboratory tests to identify male alcoholics. Am. J. Clin. Path. 82:305-310, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00241-06 LCS

PERIOD COVERED: October 1, 1984 to September 30, 1985

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

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

PI:	M. Eckardt	Section Chief	LCS, NIAAA
Other:	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 20205

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.)*

Verbal behavior is being evaluated relative to drinking history, age, and short-term and long-term abstinence in detoxified alcoholics. Specific verbal categories were determined from the content analysis of speech samples. Recent and chronic consumption variables were found to interact with each other and with age and education in a nonlinear manner in predicting impaired verbal behavior. Average impairment of verbal behavior was significantly greater among detoxified male alcoholics (N = 116) than nonalcoholics (N = 58). Alcoholics were also more impaired than nonalcoholics on measures of depression, social alienation-personal disorganization (schizoid traits), separation, guilt, diffuse anxiety, and hostility inward.

Specific verbal categories were used to predict performance on commonly used, clinical neuropsychological tests. A summary score of cognitive impairment was derived from the individual scores in various verbal categories and was found to be in the impaired range in detoxified alcoholics. Cognitive performance, as measured by analysis of verbal behavior, showed no improvement with short-term abstinence (14-20 days), but 7 months abstinence was associated with improvement.

PROJECT DESCRIPTION:

Investigators:

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

Objectives:

This study is concerned with the development of a rapidly administered neuropsychological test that can provide a large number of scores significantly similar to the test scores of many neuropsychological tests in common usage, such as the Halstead-Reitan Test Battery. These scores of cognitive functions, specifically brain function impairment, are derived from 5-minute samples of speech obtained in response to standardized and purposely ambiguous instructions. An additional goal is to determine the effects of alcoholism, age, and short-term and long-term abstinence on verbal behavior.

Methods Employed:

Subjects were male volunteers (N = 127) between the ages of 21 and 60 who were participants in a 21-day inpatient alcoholism treatment program. All were admitted with a primary diagnosis of alcoholism and had no other psychiatric disorder. Only those subjects who were capable of being tested within 7 days of their last drink and who had not taken any psychoactive medication for 48 hours prior to testing were included.

A 5-minute verbal sample was obtained at the beginning of neuropsychological testing, following the procedure of Gottschalk and Gleser (The Measurement of Psychological States through the Content Analysis of Verbal Behavior. Berkeley, Los Angeles, University of California Press, 1969). Speech samples were scored for all verbal categories of the Social-Alienation, Personal Disorganization, and Cognitive-Intellectual Impairment Scales. A battery of 24 neuropsychological tests routinely used in the clinical assessment of brain function, measuring such functions as memory, conceptual shifting, abstracting, motor performance, and general intelligence, was then administered.

This procedure was repeated after about 17 days of abstinence and again approximately 7 months after completion of treatment. A subsample of patients was re-evaluated after an additional 14 months.

Detailed information was collected and subsequently verified on alcohol consumption and on physical and mental health conditions which might affect cognition, drinking-related behavior, and socioeconomic characteristics.

Multivariate statistical approaches were used to analyze these data.

Major Findings:

Analysis of 5-minute verbal samples can be used to predict neuropsychological test performance. Cognitive functioning, as derived from analysis of verbal behavior,

is decreased in detoxified alcoholics and does not improve with 14-21 days of abstinence. Seven months of abstinence, however, is associated with improvement.

Significance to Biomedical Research and the Program of the Institute:

It has been well documented that alcoholics have impaired brain function. Neuropsychological testing is one of the most common procedures used to evaluate brain capabilities. However, these objective testing procedures require specialized training to administer and are time-consuming. Furthermore, practice effects occur when these tests are repeated on the same individuals in a relatively short period of time. The development of a rapidly administered neuropsychological test, i.e., 5-minute verbal sample, that can provide a broad and valid measure of cognition while having no demonstrable practice effect is thus of great significance. A further and more complete understanding of the verbal deficits in alcoholics might also lead to improved treatment strategies.

Proposed Course:

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

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00242-06 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Alcohol and Marijuana: Acute Effects on Cognitive Function in Humans

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

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

COOPERATING UNITS *(if any)*

Martinez Veterans' Administration Hospital, CA (R. Stillman); Department of Psychiatry, UCLA (E. Parker); Adult Psychiatry Branch, NIMH (R.J. Wyatt).

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Clinical Brain Research

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

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.)*

Acute administration of alcohol and marijuana has been shown to profoundly impair memory and cognition. Little is known about the nature of these deficits and the mechanisms underlying them. The observation that cognitively demanding tasks are particularly disrupted suggests that one way of understanding the effects of alcohol and marijuana is to investigate the types of cognitive strategies utilized when an individual is intoxicated.

The present study examines the effects of alcohol and marijuana on cognitive and memory tasks. Specific tests were selected to enable a better understanding of the cognitive strategies employed, with several of the tests being modified versions of neuropsychological tests commonly used to clinically determine brain dysfunction. It is anticipated that investigating the cognitive strategies used to solve tests which measure clinical brain dysfuncton will enable a better understanding of the detrimental effects of alcohol and intoxication on cognition.

Twenty-four male volunteers have been tested, and the data are being analyzed.

PROJECT DESCRIPTION:

Investigators:

M. Eckardt	Section Chief	LCS, NIAAA
E. Parker	Psychologist	UCLA
R. Rawlings	Mathematical Statistician	DBE, NIAAA
R. Stillman	Staff Psychiatrist	MVAH, CA
R. Wyatt	Chief	SMRA, NIMH

Objectives:

The detrimental effects of chronic alcohol abuse on cognition and memory are well known; however, the acute effects have received little systematic investigation. In the present study, commonly used neuropsychological and memory tasks were modified and administered to acutely intoxicated males. Marijuana and alcohol were administered on different days in an attempt to discern differential effects and mechanisms.

Methods Employed:

Twenty-four male volunteers were recruited, gave written informed consent, and were paid for participation. Only subjects who were judged in good health on the basis of physical and psychiatric examinations were included in the study. The dose of alcohol was 1 mL absolute ethanol/kg body weight mixed in drinks containing 3 parts fruit juice and 1 part 95% USP ethyl alcohol. Subjects drank at a steady pace for over 30 minutes. Blood alcohol concentrations were estimated by breathalyzer. In the marijuana condition, subjects smoked at a steady pace within 10 minutes a cigarette containing 15 mg THC (tetrahydrocannabinol, the active ingredient of marijuana). Prerolled, uniform marijuana cigarettes were obtained from the National Institute on Drug Abuse (2.1% THC by weight). Elevation of pulse rate was used as a physiological index of marijuana intoxication.

Modified versions of Trails A and Trails B (Reitan, R.M.: J. Consult. Psychol. 19:393, 1955) and Digit Symbol (Matarazzo, J.D.: Wechsler's Measurement and Appraisal of Adult Intelligence. Baltimore, MD, Williams and Wilkins Co., 1972) were administered four times: pre-alcohol baseline followed by alcohol one day and pre-marijuana baseline followed by marijuana on another day. The two days were spaced approximately 1 week apart, and drug order (alcohol or marijuana on day 1) was counterbalanced. Prior to test administration, eight randomly selected subjects had an apparatus fitted which enabled us to follow their eye movements while they performed the cognitive tasks.

Major Findings:

The data in this study are currently being analyzed.

Significance to Biomedical Research and the Program of the Institute:

Although the consequences of long-term alcohol abuse on neuropsychological functioning have been well documented, the acute effects are less well known. The present study employed modified versions of the same neuropsychological tests commonly used to clinically determine brain dysfunction to measure the acute effects of alcohol and marijuana on brain functioning. It is anticipated that characterization of the acute effects in terms of clinical dysfunction will enable a better understanding of the cognitive impairment which accompanies alcohol or marijuana intoxication.

Proposed Course:

Data are being analyzed. After analysis is completed, the results will be published in an appropriate scientific journal.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00247-02 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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

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

PI:	J. Johnson	Research Psychologist	LCS, NIAAA
Others:	J. Rohrbaugh	Research Psychologist	LCS, NIAAA
	D. Rio	Staff Fellow	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	Y. Davenport	Research Social Worker	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*
Behavioral Sciences Research Branch, Division of Extramural Research Programs, NIMH (J.E. Rolf)

LAB/BRANCH
Laboratory of Clinical Studies

SECTION
Section of Clinical Brain Research

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

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
3.75	2.0	1.75

CHECK APPROPRIATE BOX(ES)

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

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

Although the familial transmission of alcoholism has been well documented, very little is known about the exact mechanisms of transmission and the antecedent behaviors that lead family members to abuse alcohol. The study of persons who are most at risk for alcohol abuse (that is, the children of alcoholics) can provide information helpful for understanding this transmission.

This study attempts to characterize the behavioral and CNS developmental patterns in the children of alcoholics. First-degree relatives are also evaluated so as to compare patterns of functioning between the generations of related individuals sharing a common familial and genetic environment. The present research is designed to be a prospective, multivariate, and developmentally relevant study of both the risk and the protective factors associated with alcohol use and abuse among members of families in which at least one biological parent has been alcoholic.

PROJECT DESCRIPTION:

Investigators:

J. Johnson	Research Psychologist	LCS, NIAAA
J. Rohrbaugh	Research Psychologist	LCS, NIAAA
D. Rio	Staff Fellow	LCS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
Y. Davenport	Research Social Worker	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
J. Rolf	Psychologist	BSR, NIMH

Objectives:

Etiological factors associated with alcoholism are not well understood. This is due in part to the insufficient collection of data on persons at high risk for alcoholism. This study evaluates children at risk for alcoholism in order to identify biological, psychological, and environmental factors in order to help predict later alcoholism and to help identify patterns of interaction in the onset of the disease. Studying these children can help identify those individuals who are unlikely to develop alcoholism or those who will be unresponsive to either preventive intervention or treatment when they do develop actual alcoholic behavior. Three aims have been identified for study and analysis. The first aim is to identify, at the level of the individual, specific traits that serve as risk and protective factors for early alcohol abuse. We will attempt to replicate individual trait deficits which have been identified in the literature as potential precursors to alcohol abuse. This aim entails a univariate approach. The second aim attempts to identify, at the level of the individual, patterns of traits that serve as risk and protective factors. Here the emphasis is on a multivariate approach and an understanding of the patterns of behavior that lead individuals to abuse alcohol. The third aim is to identify variations of adaptive and maladaptive functioning among family members and between different families. Here, the unit of analysis is the family rather than the individual.

Methods Employed:

To date, a total of 180 subjects (children and adults) have been tested on an extensive psychological battery of measures which include the following dimensions: self-esteem, affect, locus of control, impulsivity/reflectivity, cognitive development, school-based competence, clinical diagnosis, and IQ. In addition, electrophysiological tests for both children and adults will be administered to measure auditory, visual, and cognitive parameters of neurophysiological development.

Major Findings:

Preliminary findings from data analyses are intriguing. Mothers' reports of their children's behavior suggest that the children of alcoholics are deemed more socially incompetent and depressed, and have more psychosomatic complaints. Although these findings are statistically significant, more reports of children are necessary to reach a definite conclusion.

A collaborative project with George Washington University was established by combining common data from our two separate but similar risk populations. Our larger sample size indicates that on measures of reflection-impulsivity and visual-spatial tasks, children of alcoholics exhibit a significantly different pattern of functioning. These data are being prepared for publication.

It is anticipated that we will collect data from approximately 80 additional subjects before statistical analysis can proceed on the project as a whole. This large number of subjects is required to enable proper multivariate statistical analysis. The assessment of the large number of dependent variables necessary in multimodal studies requires sophisticated multivariate data analytic techniques, which require a large sample size.

Significance to Biomedical Research and the Program of the Institute:

The significance of this study to biomedical research and the program of the NIAAA is to address a relatively understudied but potentially highly informative research area involving the developmental sequences through which risks for alcoholism and other maladaptive behaviors are expressed. Such studies involve age-appropriate assessments of parents and children with an integrative multimodal testing battery in which psychobiologic, psychosocial, and environmental variables are measured longitudinally.

Proposed Course:

The course of this study requires repeated testing of individuals over time with special attention to sensitive periods of adaptation when risk markers might be detected. Such longitudinal research with high-risk populations requires the establishment and maintenance of unique researcher-subject relationships. With the final implementation of the evoked potential laboratories in the Section of Clinical Brain Research, it is expected that the NIAAA will assume leadership in developmental studies in being able to relate CNS activity to a wide range of important indicators of functioning.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00250-02 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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	Medical Staff Fellow	LCS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	J. Johnson	Research Psychologist	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	H. Moss	Medical Staff Fellow	LCS, NIAAA
	D. Rio	Staff Fellow	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 20205

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.)*

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 clearly demonstrate their presence but 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) normal volunteers, in whom the effects of acute administration are examined with respect to dose-response relationships, the temporal course of effect, and relationship to blood alcohol levels; (2) social drinkers, in whom brain electrical activity is studied, in conjunction with neuropsychological data, in an attempt to assay the effects of moderate levels of alcohol consumption; and (3) abstaining alcoholics (including those with alcoholism-related mental impairment) in whom deficits are characterized, short- and long-term recovery effects (if any) during abstinence are examined, and the efficacy of various treatment strategies is examined.

PROJECT DESCRIPTION:

Investigators:

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

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 will be upon event-related brain electrical potentials, elicited in response to environmental stimulation and extracted by computer from the ongoing 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; this allows 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 in the following paragraphs.

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 period of vigilance.

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. In our studies of the effects of alcohol on orienting, we are concentrating particularly 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.

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, the distribution across sensory modalities or channels, and the 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, which is believed to be composed of early responses in primary sensory cortices, is established. 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.

Information processing stages. We believe that a powerful method 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 intensity and clarity or complexity, and assessment of the effects on speeded response performance, the stages disturbed by alcohol can be inferred. 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 poststimulus. Previous experiments have suggested that P300 is particularly important in light of data suggesting that P300 is diminished in chronic alcoholics and in their offspring as well.

Major Findings:

Previous studies have documented the usefulness of these procedures in the study of nervous system function and their sensitivity to acute and chronic alcohol consumption. To date, measures described above have been obtained from chronic alcoholics, within the context of a drug trial, on 18 experimental sessions. These data are being processed; preliminary examination confirms that the relevant potentials can be recorded from this population. Additional data have been obtained from a number of subjects for normative and pilot purposes. Subjects are presently being recruited and screened for studies of the acute effects of alcohol.

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 be of diagnostic and prognostic value in patients suffering from effects of chronic alcohol consumption.

Proposed Course:

The development of the laboratory will continue and the full complement of stimulation and recording equipment will become operational. (Computer facilities will be upgraded to allow for detailed analyses of responses, and the software will be further developed and elaborated.) Data collection will continue for ongoing studies of acute alcohol effects and will be applied to additional populations of patients and normal subjects. 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:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00236-03 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effects of Ethanol Treatment on Phenytoin Metabolism in Rats

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

PI:	E.A. Lane	Staff Fellow	LCS, NIAAA
Other:	G.F. McMorrin	Biologist	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 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

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

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

Phenytoin is a drug that exhibits undesirable side effects at concentrations just above those producing the desired anticonvulsant effect. Therefore, understanding the mechanism by which phenytoin blood concentrations are affected by alcohol consumption should improve therapy with this drug. Toward this goal, the preparation of rat liver microsomal fractions and some standard methods for evaluation of drug metabolism have been established.

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00237-03 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Evaluation of Drug-Metabolizing Status by Carbon Dioxide Breath Tests

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

PI:	E. Lane	Staff Fellow	LCS, NIAAA
Other:	I. Parashos	Visiting 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 20205

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.)*

The theoretical relationship of drug pharmacokinetics and carbon dioxide breath test results has been investigated, using the well-stirred model of liver metabolism. The effects of hepatic extraction ratio, fraction metabolized by demethylation, and absorption rate of drug upon breath test results were simulated, using the MLAB package on the DEC 10 computer. It was demonstrated that a drug suitable for detecting changes in drug-metabolizing status via the carbon dioxide breath test should be at least 50% metabolized by demethylation, should have an hepatic extraction ratio of 0.2 to 0.5, and should be administered orally in a form that is rapidly absorbed.

PROJECT DESCRIPTION:

Investigators:

E. Lane	Staff Fellow	LCS, NIAAA
I. Parashos	Visiting Fellow	LCS, NIAAA

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.

Major Findings:

Theoretically, a drug metabolized at least 50% by demethylation, having a hepatic extraction ratio of 0.2 to 0.5, and administered orally in a form that is rapidly absorbed, should be the most suitable for detecting changes in drug-metabolizing ability.

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 for rapid evaluation of drug-metabolizing ability, such as this breath test, 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. Drugs previously used (aminopyrine, caffeine, and methacetin) and another drug (imipramine) will be evaluated as probes in this liver function test.

Publications:

Lane, E.A., Guthrie, S., and Linnoila, M.: Effects of ethanol on drug and metabolite pharmacokinetics. Clin. Pharmacokin. 10:228-247, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00248-02 LCS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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	Staff Fellow	LCS, NIAAA
Other:	S. Guthrie	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 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	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.)*

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 is to be compared with the proportion of normal volunteers that exhibits the slow acetylator phenotype.

The acetylator phenotype will be measured in normal volunteers and recovered alcoholics who are not related to each other. The recovered alcoholics should have a history of alcoholism, and the normal volunteers should be nonalcoholic and have no first-degree relative who is alcoholic.

The acetylator phenotype will be determined after a single dose of sulfamethazine by measurement of blood and urine concentrations of sulfamethazine and its acetylated metabolite. These will be measured by high-pressure liquid chromatography.

The acetylator phenotype has been determined in 15 alcoholics and 10 unrelated normal volunteers. The number of subjects is insufficient to draw any conclusions regarding the relative distribution of the slow acetylator phenotype.

PROJECT DESCRIPTION:

Investigators:

E. Lane	Staff Fellow	LCS, NIAAA
S. Guthrie	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 will be measured in unrelated, age- and sex-matched normal volunteers and recovered alcoholics. The recovered alcoholics should have a history of alcoholism, and the normal volunteers should be nonalcoholics and have no first-degree relatives who are alcoholics.

The acetylator phenotype will be determined after a single dose of sulfamethazine by measurement of blood and urine concentrations of sulfamethazine and its acetylated metabolite. These will be measured by high-pressure liquid chromatography.

Major Findings:

There are no findings at this stage.

Significance to Biomedical Research and the Program of the Institute:

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; and (2) acetylation phenotype is, and alcoholism may be, genetically determined, and the expression of alcoholism may depend on genetic factors related to acetylation.

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

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00255-01 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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. Lane	Staff Fellow	LCS, NIAAA
Others:	S. Guthrie	Senior Staff Fellow	LCS, NIAAA
	I. Parashos	Visiting Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	H. Moss	Medical 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 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.8	0.8	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The disposition in human patients of 3-methoxy-4-hydroxy phenylglycol (MHPG), a major metabolite of norepinephrine, has been modeled. The cerebrospinal fluid concentration of MHPG and the urinary excretion rates of its metabolites before and during treatment with desipramine (DMI) and zimelidine (ZIM) were used to evaluate the effect of drug treatment upon the central nervous system rate of production of MHPG. The central rate of production of MHPG was decreased ($P < .05$) by 45.7 $\pm$ 17.4% by DMI treatment and by 21.4 $\pm$ 17.9% by ZIM treatment.

A well-stirred model of liver metabolism has been applied to published data on the disposition of melatonin in humans. Melatonin can be described as a substance subject to a large first pass effect of hepatic metabolism. This first pass effect does not appear to account for all of the poor oral bioavailability of melatonin. Although it has been reported that the elimination rate of melatonin is decreased in patients with hepatic cirrhosis, the application of clearance concepts to the same data provides evidence that the endogenous production rate of melatonin is also decreased.

PROJECT DESCRIPTION:

Investigators:

E. Lane	Staff Fellow	LCS, NIAAA
S. Guthrie	Senior Staff Fellow	LCS, NIAAA
I. Parashos	Visiting Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
H. Moss	Medical Staff Fellow	LCS, NIAAA

Objectives:

Studies will be designed to elucidate the in vivo disposition of neurotransmitters and their metabolites in animals and humans and the effects of various treatments upon that disposition. This may lead to new hypotheses about the biochemical bases of psychiatric disorders and the mechanisms of action of drugs used in treatment.

Methods Employed:

The disposition of neurotransmitters and their metabolites is modeled according to available knowledge, including that of metabolic pathways and the sites of metabolism. Studies are then designed to fill in the unknown factors of the model. These studies include administration of neurotransmitters and metabolites to animals and humans and analysis of these compounds and metabolites in various biological fluids. The administered substances are usually labeled with radioactive or stable isotopes. The analyses are accomplished by high-pressure liquid chromatography, gas-liquid chromatography, and mass spectrometry.

Major Findings:

The disposition in human patients of 3-methoxy-4-hydroxy phenylglycol (MHPG), a major metabolite of norepinephrine, has been modeled. The cerebrospinal fluid concentraton of MHPG and the urinary excretion rates of its metabolites before and during treatment with desipramine (DMI) and zimelidine (ZIM) were used to calculate the effect of drug treatment upon the central nervous system rate of production of MHPG. The central rate of production of MHPG was decreased ($P < .05$) by $45.7 \pm 17.4\%$ by DMI treatment and by $21.4 \pm 17.9\%$ by ZIM treatment.

A well-stirred model of liver metabolism has been applied to published data on the disposition of melatonin in humans. Melatonin can be described as a substance subject to a large first pass effect of hepatic metabolism. This first pass effect does not appear to account for all of the poor oral bioavailability of melatonin. Although it has been reported that the elimination rate of melatonin is decreased in patients with hepatic cirrhosis, the application of clearance concepts to the same data provides evidence that the endogenous production rate of melatonin is also decreased.

Significance to Biomedical Research and the Program of the Institute:

Rational interpretation of in vivo measurements of neurotransmitters and their metabolites will help clarify any neurochemical aspects of alcoholism.

Proposed Course:

We propose to design and carry out studies to determine what fraction of the total daily production of the norepinephrine metabolite (MHPG) is produced in the central nervous system.

Publications:

Linnoila, M., Lane, E.A., Guthrie, S., Parashos, I., Rudorfer, M., Potter, W.Z.: CSF, plasma and urine. What do concomitant measurements of norepinephrine and its metabolites mean? Psychopharm. Bull. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00235-03 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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)*

PI:	N. Salem, Jr.	Research Chemist	LCS, NIAAA
Others:	J. Yergey	Senior Staff Fellow	LCS, NIAAA
	H. Kim	Visiting Fellow	LCS, NIAAA
	T. Shingu	Visiting Fellow	LCS, NIAAA
	M. Stojanov	Visiting Fellow	LCS, NIAAA
	D. Melville	Chemist	LCS, NIAAA
	R. Whitmore	Biological Lab. Technician	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 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.1	2.6	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.)*

The principal objectives of this study are to elucidate the structural and metabolic functions of polyunsaturated fatty acids or phospholipids with particular reference to their modulation by ethanol. To this end, we investigated novel routes and sources of oxygenated metabolites of the major polyunsaturate of the brain, docosahexaenoic acid (22:6). Chromatographic, mass spectrometric, and extraction techniques were developed in order to characterize C22:6 products enzymatically formed by rat brain homogenate in vitro. We observed several mono-, di-, and trihydroxylated products which have not been previously described. These structural identifications were obtained after HPLC-thermospray MS in the positive ion mode and negative ion thermospray analysis of the pentafluorobenzyl derivatives of purified peaks. In combination with organic reactions such as hydrogenation and methoximation, trihydroxy-22:6 and -22:5, dihydroxy-22:5, and monohydroxy-22:6 peaks were assigned. The synthesis of these leukotriene-like products is stimulated by low concentrations of ethanol.

Progress in analyzing the structural functions of unsaturated lipids was made by the development of rapid and efficient phospholipid molecular species analysis by HPLC-thermospray. Simple and information-rich fragmentation patterns, which readily yield head group and fatty acyl composition, were observed. This analytic technique has been successfully applied to complex biological mixtures for choline, ethanolamine, and serine phosphoglycerides.

PROJECT DESCRIPTION:

Investigators:

N. Salem, Jr.	Research Chemist	LCS, NIAAA
J. Yergey	Senior Staff Fellow	LCS, NIAAA
H. Kim	Visiting Fellow	LCS, NIAAA
M. Stojanov	Visiting Fellow	LCS, NIAAA
T. Shingu	Visiting Fellow	LCS, NIAAA
R. Whitmore	Biological Lab. Technician	LCS, NIAAA
D. Melville	Chemist	LCS, NIAAA
M. Engler	Graduate Student	Georgetown Univ.
C. Masters	Biological Aide	LCS, NIAAA

Objectives:

(1) To discover and characterize a novel metabolic system of lipoxygenase and/or cyclooxygenase enzymes in the mammalian brain, which operates on W-3 fatty acids.
(2) To elucidate the molecular composition, associations, and topographic arrangement of polyunsaturated phospholipids in the plasma membrane.
(3) 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.

Methods Employed:

Rat brain homogenate was incubated with [^{14}C]-22:6 fatty acid and centrifuged; ethanol was added to the supernatants to make a 15% solution. Various lipid classes were then separated, using a liquid-solid extraction technique (Sep-Pak cartridges). The oxidized metabolites were eluted with ethyl acetate after washing with 15% ethanol and benzene. Chromatographic separation was then obtained by high-pressure liquid, thin layer, or gas-liquid chromatography after suitable derivatization reactions. Enzymatic production of oxidized 22:6 derivatives was monitored by fraction collection and liquid scintillation counting, by autoradiography, or by use of a flow-through beta counter (Radiomatic). A variety of mass spectrometric sample introduction techniques were employed, including thermospray, GC-MS, and desorption chemical ionization.

Phospholipids were extracted from tissues using chloroform-methanol-water as described by Bligh and Dyer. Fatty acids were transmethylated and quantitated by GC-MS in the electron impact mode. Alternatively, phospholipid classes were subfractionated by reversed-phase HPLC using methanol-hexane-0.1 M ammonium acetate as the mobile phase. Separated molecular species were ionized in the thermospray probe at 148° C with assistance from the auxiliary filament and extracted into a quadrapole mass filter for detection.

Major Findings:

A new HPLC separation system was developed for separation of mono-, di-, and trihydroxylated 22:6 fatty acid metabolites formed by brain tissue in vitro. Ultraviolet spectra of these peaks indicated that they were conjugated dienes or

trienes as they absorbed at 237 or 275 nm. This HPLC technique was used for on-line thermospray mass spectral analysis of these oxygenated metabolites. Spectral analysis indicated that several di- and trihydroxy products were present, including trihydroxy 22:5 and 22:6 and a dihydroxy 22:5; only a single monohydroxy peak with six double bonds was observed. The molecular weights of 378, 362, and 344 for the respective compounds were obtained by analysis of the pentafluorobenzylesters in the negative ion mode. Preliminary data indicate that biosynthesis of these products is stimulated by low ethanol concentrations but is unaffected by indomethacin, a potent cyclooxygenase inhibitor.

Thermospray mass spectrometry proved to be a very effective tool for compositional analysis of phospholipid molecular species as well. A simple fragmentation pattern was observed, which included the molecular, diglyceride, fatty acyl, and head group ions. Thus, all of the important information can be rapidly obtained following a short chromatographic run. Egg choline or ethanolamine phosphoglycerides were subfractionated into 10 molecular species in 15 or 30 minutes, respectively. Brain phosphatidylserine could be resolved into six molecular species in only 6 minutes. These methods can be readily generalized for other lipid derivatives and classes.

Significance to Biomedical Research and the Program of the Institute:

We have described an enzymatic system not previously observed in the brain or any mammalian tissue that is analogous to the lipoxygenase/leukotriene system present in the periphery. Although the function of this product is unknown, its similarity to the leukotriene system leads us to hypothesize a potent hormonal-like action in the nervous system. The elevation of its level and the previously described decrease in its precursor induced by alcohol may indicate an important role for this system in the mechanism of action of ethanol in the brain. It is likely that these products are members of an entirely new family of bioactive compounds.

The development of rapid and efficient methods for phospholipid molecular species analysis will allow the detailed examination of altered lipid metabolism and membrane structure resulting from alcohol exposure.

Proposed Course:

We will apply the latest technology in chromatography and analysis in order to purify these compounds to homogeneity. We will use these analytic criteria to optimize the biochemical conditions required for maximal product yield. These conditions will then be used to prepare milligram quantities of the oxygenated metabolites of the 22:6 fatty acid, using semi-preparative HPLC. The material will be used for a complete structure determination including hydrogenation and ozonolysis for determination of hydroxy and double bond positions and number, accurate mass GC-MS, and high resolution NMR analysis. The material will also be used for physiological studies on platelets and vascular smooth muscle preparations as well as for a broad survey of biochemical functions of the pre- and postsynaptic membranes in the nervous system. The ethanol modulation of the enzymatic system will be carefully described, and the presence of the metabolite in human tissues in vivo will be investigated as a basis for further studies of its pathophysiology in alcoholic patients.

The thermospray analysis of phospholipids will be generalized to other phospholipid classes and applied to the quantitative analysis of red blood cell, platelet, and serum species. Similar methods for the dinitro- and trinitrophenyl derivatives will make possible membrane molecular species asymmetry analysis. These assays will be applied to human alcoholics and to the study of alterations in membrane structure and function in tissues from rats exposed to alcohol through an inhalation technique.

Publications:

Kim, H.Y., Pilosof, D., Kyches, D.F., and Vestal, M.L.: On-line peptide sequencing by enzymatic hydrolysis, high performance liquid chromatography and thermospray mass spectrometry. J. Am. Chem. Soc. 106:7304, 1984.

Bougnoux, P., Salem, N., Jr., Lyons, C., and Hoffman, T.: Alteration in the membrane fatty acid composition of human lymphocytes and cultured transformed cells induced by interferon. Molecular Immunology (in press).

Salem, N., Jr., Kim, H.Y., and Yergey, J.A.: Docosahexaenoic acid: membrane structure and function. In Lands, W.E.M., Leaf, A., Grundy, S.M., Austin, K.F., and Salem, N., Jr. (Eds.): Health Effects of Polyunsaturated Fatty Acids in Seafoods. New York: Academic Press (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00251-02 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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.	Research Chemist	LCS, NIAAA
Others:	J. Karanian	Senior Staff Fellow	LCS, NIAAA
	M. Stojanov	Visiting Fellow	LCS, NIAAA
	D. Melville	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 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.9	1.1	0.8

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The objective was to extend our data base to include the effects of an in vivo ethanol exposure paradigm on vascular reactivity with particular reference to the involvement of the prostaglandin system in such effects. The design and setup of inhalation chambers and a monitor/control apparatus for an ethanol vapor exposure paradigm has allowed us to examine the cardiovascular consequences of graded blood ethanol concentrations (BEC) during acute and chronic exposure periods. For this purpose, (1) a smooth muscle bath system was set up to measure reactivity of isolated vasculature, using force displacement transducers, and (2) a harness and tether system was set up in order to maintain a cannula in ambulatory rats while measuring changes in blood pressure (systemic arterial pressure) and its reactivity, using pressure transducers. Our in vitro results in the rat aorta, following either short-term (1-day) or long-term (14-day) exposure to ethanol vapor, show a biphasic response with regard to the contractile response to a thromboxane-mimic; moderate BEC decreased contractility, whereas high levels had no effect. Similarly treated rats showed a marked increase in the PGI-2/TXA-2 ratio, due primarily to increased PGI-2 levels, which coincided with a depression in contractility. PGI-2 levels were not affected in aorta obtained from rats with high BEC. Chronic exposure to ethanol vapor resulted in a similar PGI-2/TXA-2 ratio but lower absolute levels of these prostaglandins, which may have resulted from arachidonate depletion.

Preliminary studies indicate that blood pressure and its reactivity is related to the BEC in these rats. Acute exposure to physiologically tolerable BEC depressed blood pressure and was directly related to the time of exposure to ethanol vapors. However, the BP of high-salt rats increased after 6 days of ethanol exposure. Vasoreactivity was enhanced in normal rats following a similar exposure to ethanol vapors; both vasopressor and vasodepressor effects were markedly potentiated.

PROJECT DESCRIPTION:

Investigators:

N. Salem, Jr.	Research Chemist	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA
D. Melville	Chemist	LCS, NIAAA
M. Engler	Graduate Student	Georgetown Univ.

Objectives:

To elucidate the mechanism by which alcohol affects vascular tonus and contractility and, in particular, to define the role of prostaglandins and other polyunsaturated fatty acid metabolites in these effects.

Methods Employed:

Two types of alcohol inhalation chambers were designed and constructed for either chronic exposure (type A) or in vivo blood pressure monitoring (type B). An ethanol vaporization and metering apparatus with chamber ethanol concentration sensing and feedback control was designed.

Male Sprague-Dawley rats (350-400 g) were a heterogeneous population with regard to BEC when exposed to the same ethanol vapor concentration (24.8 mg/L). A gaussian-like distribution was observed with 4.7% of the population below 50 mg% and 18.1% above 350 mg%, of which 13.4% die. BEC were determined enzymatically after bleeding rats through the tail artery or vein. Rats were housed in chamber type A for short- and long-term exposure periods, and (1) rings were cut from thoracic aorta and fixed between hooks in a 40 mL bath containing Krebs-Ringer Bicarbonate maintained at 37° C and equilibrated with a mixture of 95% O_2 and 5% CO_2 at a tension of 1 gm, and the hook anchoring the upper end of the ring was connected to the lever of a force displacement transducer; or (2) rings were similarly prepared and incubated in buffer for the purpose of obtaining samples for determination of endogenous levels of PGI_2 and TXA_2 by RIA.

Alternately, rat tail arteries were cannulated and connected through a harness to a tether and swivel system in chamber type B for chronic blood pressure monitoring in the awake animal. Blood samples were obtained from the intraarterial line and collected in syringes containing EDTA (1 mg/mL) and indomethacin (10 g/mL). Plasma was immediately obtained (1000 x g x 1 min) and frozen at -70° C for future determination of plasma PGI_2 and TXA_2 levels with RIA. Dose-response curves for the vasoactive agents noradrenaline, U46619, arachidonic acid, PGI_2, and carbachol were obtained by administering graded doses of each agent into the smooth muscle bath (in vitro studies) or via the cannula (in vivo studies). Lipids were extracted from aortas for fatty acid analysis by gas chromatography following transmethylation.

Major Findings:

Physical dependence was induced in rats during exposure to ethanol vapors. Behavioral signs of withdrawal from alcohol (body and tail tremors, rigidity, convulsions) were observed within a few hours after removal from the inhalation

chambers and lasted up to 24 hours following exposure periods of 3-14 days. Hypoactivity (grid crossings, head bobbings, etc.) was observed after a 24-hour exposure period to ethanol vapors.

In vitro studies. Following a short-term (1-day) or long-term (14-day) exposure to 60-200 mg% BEC, the maximum response of isolated rat aorta to a TXA_2-mimic significantly decreased. In contrast, contractility of aorta from rats with BEC greater than 200 mg% were not affected. Similarly exposed rats having moderate BEC showed marked stimulation of the net PGI_2 synthesis rate; however, the rate of TXA_2 synthesis decreased. Thus, an increase in the PGI_2/TXA_2 ratio coincided with a depression in contractility. Net PGI_2 synthesis rate was unaffected but the TXA_2 levels were depressed in aorta obtained from rats with high BEC. The long-term alcohol exposure period markedly depressed the prostaglandin levels. A 14-day exposure to ethanol vapors decreased arachidonic acid (20:4) content in the total lipid extract of cardiovascular tissue; 50% and 23% decreases were observed in the aorta and the heart, respectively.

In vivo studies. Systemic arterial pressure was inversely related to the time of exposure to ethanol vapors (158-380 mg%); 7%, 11%, and 34% decreases were observed at 60 minutes, 6 hours, and 6 days, respectively. Blood ethanol concentrations greater than 120 mg% induced 8.3% and 49% increases in the systemic arterial pressure of high-salt rats compared to normal-fed and pair-fed rats, respectively. Vasoreactivity was enhanced following long-term exposure of normal rats to ethanol vapors. The maximum vasopressor response to noradrenaline (1.8 μg/kg) and U46619 (9.6 μg/kg) was potentiated 107% and 43%, respectively, and the vasodepressor effect of both graded doses of carbachol (3.6, 7.2, 14.4, and 28.8 μg/kg) was increased by 151%, 85%, 52%, and 24%, respectively, after similar ethanol exposure.

Significance to Biomedical Research and the Program of the Institute:

We are investigating the proposition that endogenous synthesis rates of prostaglandins such as PGI_2 and TXA_2 in vascular tissue are related to alcohol consumption and associated with changes in blood pressure and its reactivity. Our data indicate that physiological levels of alcohol induce an increase in the PGI_2/TXA_2 ratio that is concomitant with a depression in contractility in rat aorta. These data are consistent with the hypothesis that moderate consumption of alcohol could shift the homeostatic balance toward platelet antiaggregatory-vasodilatory processes in vivo. This observation may have significance in the elucidation of the mechanism by which moderate alcohol consumption could protect against atherosclerosis-related cardiovascular disease. Chronically high BEC enhanced vasoreactivity, whereas blood pressure was depressed. Permutations such as a high-salt diet may increase sensitivity to alcohol-related hypertension. We expect that the relationship between endogenous prostaglandin levels and blood pressure and its reactivity may in part explain the effect of alcohol on cardiovascular homeostasis. Chronic alcohol exposure increased sensitivity to vasoactive agents that are involved in blood pressure regulation. These results may provide a mechanism associated with the higher incidence of hypertension in alcoholics.

Proposed Course:

Our data base is currently being extended to include the effects of various blood alcohol levels on blood pressure, pressor and depressor responses, and related changes in fatty acid and prostaglandin levels. Specific enzymatic inhibitors and receptor antagonists will be used to tease out the role of endogenously produced prostaglandins in alcohol-induced cardiovascular changes. The alcohol-exposure paradigm will allow us to examine the cardiovascular consequences of acute and chronic exposure in a series of permutations involving dietary manipulation of essential fatty acid levels (both omega 3 and 6 series) and salt content in normotensive and spontaneously hypertensive rats. These results will be used for formulation of carefully defined clinical protocols.

Publications:

Karanian, J.W., Stojanov, M., and Salem, N., Jr.: Effect of ethanol on prostacyclin and thromboxane A_2 synthesis in rat aortic rings *in vitro*. *Prostaglandins and Leukotrienes in Medicine* (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA00262-01 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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.	Research Chemist	LCS, NIAAA
	H. Kim	Visiting Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA
	D. Melville	Chemist	LCS, NIAAA
	R. Whitmore	Biological Lab. Technician	LCS, NIAAA

COOPERATING UNITS *(if any)*

Clinical Neuroscience Branch, NIMH (A. Durand); Neuropsychiatry Branch, St. Elizabeth's Hospital (C. Kaufmann).

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Analytical Chemistry

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.)*

The principal objective of this project is to apply state-of-the-art gas chromatographic/mass spectrometric methodologies to the analysis of trace levels of fatty acid metabolites in an effort to elucidate the molecular alterations involved in various neuropathological states. These compounds are generally potent agents that are present in extremely low levels or in such complex mixtures that other techniques often cannot provide reliable results. During the first phase of this research, emphasis has been placed on characterizing the levels of prostaglandins in human cerebrospinal fluid (CSF).

Preliminary investigations have suggested that prostaglandin levels in CSF drawn from patients with neurological impairment are markedly altered from those of normal volunteers. The levels are often near or below the reported detection limits of 10-100 pg/mL of CSF. The results described in this study summarize the methodologies used to demonstrate significantly lower levels of detection for application to the analysis of CSF samples from a variety of patient groups. Of particular importance is the application of these methodologies for the first time to samples of CSF drawn from alcoholic patients during withdrawal.

PROJECT DESCRIPTION:

Investigators:

J. Yergey	Senior Staff Fellow	LCS, NIAAA
N. Salem, Jr.	Research Chemist	LCS, NIAAA
H. Kim	Visiting Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
D. Melville	Chemist	LCS, NIAAA
R. Whitmore	Biological Lab. Technician	LCS, NIAAA
A. Durand	Medical Staff Fellow	NSB, NIMH
C. Kaufmann	Medical Staff Fellow	St. Elizabeth's Hospital

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 the analysis of trace oxygenated fatty acid metabolites in various biological fluids; and (3) to develop an assay for the analysis of prostaglandins in cerebrospinal fluid with the goal of correlating their levels with the clinical state of the patient.

Methods Employed:

Samples of 2 mL cerebrospinal fluid (CSF) were acidified to pH 3.5 and applied to pre-soaked Sep-Pak ODS cartridges. Elution with 20 mL each of 15% ethanol, benzene, and ethyl acetate effectively isolated the prostaglandins. The ethyl acetate fraction, containing the prostaglandins, was blown to dryness and derivatized to form the pentafluorobenzyl (PFB) ester, methoxime, trimethyl silyl ether for the prostaglandins. The PFB ester is a highly electronegative species which is capable of efficient electron capture in a mass spectrometer ion source operated in negative chemical ionization mode. Derivatized samples were analyzed by on-column capillary gas chromatography/negative chemical ionization mass spectrometry. The mass spectrometer was operated in selected ion mode, i.e., only the masses corresponding to the major ion for each prostaglandin were monitored, thus greatly increasing the sensitivity of the technique. Quantification of the prostaglandins is made by comparison to signals for tetradeuterated standards added to the sample.

Major Findings:

The sample preparation methodology developed is an adaptation of reported procedures which we have optimized for sensitivity in analyzing prostaglandins in CSF. Sample losses were minimized by reducing sample isolation to a single Sep-Pak extraction followed by derivatization in a single vial. Eliminating additional sample extraction and chromatographic steps seemed a reasonable approach for CSF samples, which are inherently cleaner than urine or plasma. Further sensitivity increases were realized by utilizing cold on-column injection in a capillary gas chromatographic separation of the derivatives. Prostaglandins eluting from the gas chromatographic oven at 270°C were baseline separated in most cases. Since

each ion monitored by the mass spectrometer corresponds to a single prostaglandin, partially overlapping chromatographic peaks can be separately identified by the mass spectrometer. The sensitivity of this method is derived from the electron-capturing abilities of the PFB ester of the compound, which yields an intense [M-PFB]- ion in the mass spectrometer. Limits of detection for standards approached 1 femtomole per mL CSF, which corresponds to 60 fg of compound injected on-column. Initial results from the CSF of a human patient demonstrated the ability to recover the deuterated internal standard from the sample and showed evidence of the presence of PGF2 alpha and 6-keto PGF1 alpha in the CSF.

Significance to Biomedical Research and the Program of the Institute:

The sensitivity and selectivity inherent in this type of mass spectrometric measurement make possible the simultaneous detection of trace levels of several oxygenated fatty acid metabolites in cerebrospinal fluid. These compounds are extremely potent bioregulatory agents involved in modulating the activity of nearly every organ in the body. Ethanol has the capability of interfering with their metabolism, thereby leading to a pathological state in many organ systems, including the brain. We expect that characterization of these compounds should lead to an understanding of abnormal brain metabolism in alcoholics as well as in patients with other mental disorders.

Proposed Course:

Additional study will be made of the sample workup procedure in order to obtain complete recovery of prostaglandins from CSF samples. Several minor modifications to the mass spectrometric instrumentation are in progress which should also further improve the sensitivity of the technique. The methods developed will be applied to CSF samples collected from patients with schizophrenia, affective disorders, and alcoholism. These data will be analyzed in an attempt to correlate the prostaglandin levels in the cerebrospinal fluid with each diagnostic group. The analytical methodologies will also be extended to include simultaneous quantification of lipoxygenase products (i.e., the leukotrienes) as well as other metabolites and body fluids.

Publications:

Yergey, J.A., Cotter, R.J., Heller, D., and Fensdau, C.: Resolution requirements for middle molecule mass spectrometry. Anal. Chem. 56:2262-2263, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00243-02 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Influence of Ethanol and Glucocorticoids on GABA Receptors in the CNS

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

PI:	R. Eskay	Research Physiologist	LCS, NIAAA
Others:	M. Majewska	Visiting Associate	LCS, NIAAA
	J. Bisserbe	Visiting Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

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 exerts a dose-dependent stimulatory or inhibitory effect on the CNS; the mechanism of these effects is not, however, clear. Ethanol alters the action of several centrally acting GABA-ergic drugs, such as benzodiazepines and barbiturates, which suggests that ethanol interacts with GABA receptors. Ethanol exerts a biphasic effect on the binding of muscimol (GABA-A agonist) to GABA receptors in synaptosomal membranes obtained from different brain regions. Muscimol binding is inhibited at concentrations of ethanol ranging from 1.5 mM to 6 mM, whereas it is enhanced at higher concentrations. Decreased muscimol binding in the presence of low levels of ethanol was found to be due to reduced ligand affinity to the GABA receptors, whereas enhanced muscimol binding at high ethanol concentrations was due to increased receptor number.

Glucocorticoids also appear to act directly on central GABA receptors, but in a dose-dependent manner opposite to that of ethanol. Nanomolar concentrations of glucocorticoids enhance muscimol binding, whereas micromolar concentrations inhibit binding. These effects are due to increased receptor affinity for the ligand and a decreased receptor number in the presence of low or high levels of glucocorticoids, respectively.

This project has been completed.

PROJECT DESCRIPTION:

Investigators:

R. Eskay	Research Physiologist	LCS, NIAAA
M. Majewska	Visiting Associate	LCS, NIAAA
J. Bisserbe	Visiting Fellow	LCS, NIAAA

Objectives:

The specific aims of this study were to evaluate the direct effect of ethanol on muscimol ($GABA_A$ agonist) binding to GABA receptors in the CNS and to determine the mechanism of action of ethanol on central GABA receptors.

Methods Employed:

Tissue preparation. Sprague-Dawley (200-250 g) rats were killed by decapitation, and their brains were dissected into various regions, using the Glowinski and Iversen method. Crude synaptosomal fractions were obtained from each brain region by differential centrifugation of brain homogenates in 0.32 M sucrose. Membranes were prepared by hypo-osmotic shock, polytron homogenization, and centrifugation at 12,000 xg. Membrane preparations were resuspended in 50 mM Tris-HCL, pH 7.4, frozen, thawed, and washed four times with cold buffer.

Binding of muscimol. Muscimol binding ($GABA_A$ receptor agonist) was studied in membranes from the crude synaptosomal fraction prepared from various brain regions. Membranes were incubated with [H^3]-muscimol at 37° C for 10 minutes in 50 mM Tris-HCL, pH 7.4, containing 1 mM dithiotreitol. Nonspecific binding was determined in the presence of 1 mM GABA. The binding reaction was terminated by centrifugation, and the pellet was washed with buffer and counted.

The effect of ethanol on muscimol binding was studied by the addition of 0.22 mM to 1.5 M ethanol into the incubation mixture. The effect of corticosteroids on muscimol binding was examined by the addition of endogenous (corticosterone and pregnenolone-SO_4) or synthetic (6-methyl-prednisolone and dexamethasone) steroids (10^{-10} to 10^{-4} M) to the binding mixture.

Autoradiographic muscimol binding. Brain sections (20 microns thick) were obtained from normal and adrenalectomized rats and mounted on gelatin-coated glass slides. Two sets of adjacent sections were obtained from various regions of the rat brain to determine total and nonspecific muscimol binding. Binding studies were performed as follows: 20-minute preincubation in 50 mM Tris-HCL buffer, pH 7.4, containing 1 mM dithiotreitol; 20-minute incubation in 50 mM Tris-HCL buffer containing 6 nM [^{3}H]-muscimol with or without steroids (pregnenolone-SO_4, 10^{-8} M); 40-second wash; drying under cold air stream. Matching sets of sections were then placed in apposition to LKB film in a light-proof cassette and the film was processed using D19 (4 minutes) rapid fix (Kodak 2 minutes).

Major Findings:

Ethanol alters ligand binding to GABA receptors in the CNS in a biphasic manner. In certain brain areas, such as cerebellum, cortex, hippocampus, and olfactory

bulbs, ethanol at low concentrations (1.5-6 mM) decreases muscimol binding, whereas ethanol at higher concentrations increases muscimol binding to control levels and above. Similar effects were not observed in the hypothalamus. Scatchard analysis of the binding data suggests that the decrease in muscimol binding at low ethanol concentrations was due to decreased ligand affinity of GABA receptors, and the enhancement of muscimol binding at higher alcohol concentration was due to an increase in the muscimol binding capacity. The reduction of muscimol binding observed with low concentrations of ethanol (up to 6 mM) is similar to the effect of adrenalectomy (Adx). Adx reduced ligand binding to GABA receptors in several brain regions, such as cortex, hippocampus, cerebellum, and thalamus, but not in the hypothalamus or pons-medulla region. Adx-reduced muscimol binding is due to a decrease in the affinity of GABA receptors for the ligand. These affinities can be restored to control levels or above by the addition of corticosteroids, such as corticosterone, pregnenolone-SO_4 or the synthetic steroid 6-methyl-prednisolone (given in order of decreasing potency) but not by dexamethasone. The effect of glucocorticoids on muscimol binding was biphasic with physiological (nanomolar) concentrations of steroids stimulating muscimol binding due to an increased affinity of the receptors for the ligand, and supraphysiological concentrations (micromolar) of steroids inhibiting muscimol binding due to a reduction in the GABA receptor number. Test-tube and autoradiographic binding methods have yielded compatible results.

Physiological levels of plasma glucocorticoids appear to act as endogenous modulators of GABA receptors, which suggests that glucocorticoids may function as natural anticonvulsants. The potentiation by glucocorticoids of the inhibitory function of GABA receptors may prevent neuronal overstimulation during stress. Since alcohol is known to remove some steroids from cellular membranes, it may express its effect on GABA receptors by preventing the binding of or incorporation of steroid hormones to neuronal membranes; therefore, the presence of ethanol may have an effect similar to Adx. Our data suggest that the interaction of GABA receptors with alcohol or corticoids may occur via a common, competitive mechanism of action. Considering the fundamental role of the inhibitory GABA-ergic system in the overall function of the CNS, it is possible that the glucocorticosteroid-GABA receptor interaction may be of fundamental physiological importance, and the presence of ethanol may alter this interaction. It is of particular interest to note that many alcoholic patients have an abnormally functioning hypothalamic-pituitary-adrenal axis, particularly during withdrawal from ethanol, coinciding with an increased risk of seizures.

Significance to Biomedical Research and the Program of the Institute:

The neurotropic effects of ethanol may be related to the direct effect of ethanol on central GABA receptors, and therefore the impact of ethanol on all GABA-ergic drugs (in particular, the benzodiazepines and barbiturates) could be anticipated.

Proposed Course:

This project as a separate entity has been completed.

Publications:

Majewska, M.D., Bisserbe, J.C., and Eskay, R.L.: Glucocorticoids are modulators of $GABA_A$ receptors in brain. Brain Research (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00244-02 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Ethanol-Induced Changes in B-Endorphin and CRF Binding to Peripheral Tissue

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

PI:	R. Eskay	Research Physiologist	LCS, NIAAA
Others:	J. Dave	Visiting Associate	LCS, NIAAA
	J. Karanian	Senior Staff Fellow	LCS, NIAAA
	K. Mishler	Biological Lab. Technician	LCS, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Cell Biology, NIMH (L. Eiden)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
1.5	1.3	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.)*

Although ethanol is commonly regarded as an anxiolytic, its administration mimics many actions of stress that involve activation of the hypothalamic-pituitary-adrenal axis (HPAA). An understanding of the effect of ethanol on this axis requires an appreciation of the central and peripheral regulatory factors and their interactions, which constitute the HPAA. Intimately involved in the homeostasis of the HPAA are several neuropeptides of brain origin (vasopressin and corticotropin-releasing factor [CRF]) and pituitary origin (beta-endorphin [BE] and adrenocorticotropin). The long-term goal of these studies is to understand the effect of ethanol at each level of the HPAA, with special emphasis on neuropeptide-dependent events. Before evaluating ethanol-induced perturbations of CRF and BE, it was necessary to validate the existence of specific BE and CRF binding sites in peripheral tissues, and to determine if occupancy of these receptors altered any of the known second-messenger systems. We have (1) identified, using 125-I-rCRF, specific binding sites for CRF in various rat peripheral tissues and bovine chromaffin cells in culture and (2) established that occupancy of the CRF binding sites, at least in rat adrenal membranes and bovine chromaffin cells, activates the adenylate cyclase/cAMP system. Furthermore, we have (1) identified, using 125-I-human BE, specific binding sites for BE in various rat peripheral tissues and (2) established that occupancy of the BE binding sites, at least in rat hepatic membranes, activates the adenylate cyclase/cAMP system. Exposure of rats to ethanol vapor for 14 days lowered immunoreactive plasma BE levels and reduced CRF and BE binding to a variety of peripheral tissues. In addition, chronic exposure of rats to ethanol vapors lowered pituitary CRF binding, basal and CRF-stimulated adenylate cyclase activity, and pro-opiomelanocortin messenger RNA levels.

PROJECT DESCRIPTION:

Investigators:

R. Eskay	Research Physiologist	LCS, NIAAA
J. Dave	Visiting Associate	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA
K. Mishler	Biological Lab. Technician	LCS, NIAAA
L. Eiden	Senior Staff Fellow	LCB, NIMH

Objectives:

The specific aims of this study were to determine if chronic ethanol treatment of rats (1) modified nonopiate BE binding to liver and kidney membranes, (2) altered BE binding to hepatic and kidney membranes following in vitro ethanol treatment, (3) altered CRF binding, basal and CRF-stimulated adenylate cyclase activity, and pro-opiomelanocortin (POMC) mRNA levels in the anterior and neurointermediate lobes of the pituitary gland, and (4) altered circulating immunoreactive BE levels.

Methods Employed:

Tissue preparation. Animals were decapitated, and various peripheral tissues and anterior and intermediate lobes of the pituitary gland were immediately frozen in liquid nitrogen and processed for various assays as previously described.

Iodination of peptides. Acetyl-human B-endorphin or rat CRF was iodinated with ^{125}I (Amersham) by a modification of the lactoperoxidase method of Thorell and Johansson as previously described.

B-endorphin and CRF binding assays. These assays were carried out as previously described (see reference).

Adenylate cyclase assay. The standard assay system for the determination of adenylate cyclase activity was essentially as described by Cote et al. (Endocrinol. 107:108, 1980), with the exception that the amount of cAMP formed was determined using an NEN kit (New England Nuclear, Boston, MA).

POMC mRNA analysis. The anterior or neurointermediate lobe of the pituitary gland was processed as previously described, and mRNA levels were quantitated by Northern-blot and slot-blot techniques.

Major Findings:

Validation of the existence of specific BE binding sites in peripheral tissue and ethanol-induced changes in BE binding. Using ^{125}I-acetyl-human B-endorphin, specific binding sites for BE were found in the liver, kidney, adrenal, spleen, testis, and erythrocytes of adult male rats. Binding was saturable, and maximal binding was achieved with a 60-minute incubation at 22° C. In addition, incubation of hepatic microsomal membranes with BE induced a dose-related increase in membrane adenylate cyclase activity. Acute in vitro exposure of hepatic and kidney membranes to ethanol resulted in a dose-related enhancement of BE binding with maximal binding observed at 0.2% ethanol concentration. In contrast, chronic

ethanol treatment in vivo decreased ^{125}I-BE binding to hepatic, kidney, and erythrocyte membranes by approximately 35-40%, which was due to a decrease in the number of BE binding sites. The addition of ethanol in vitro to these membranes derived from rats chronically exposed to ethanol in vivo did not further alter BE binding, which suggests that chronic ethanol exposure induces compositional membrane changes.

Characterization of specific CRF binding sites in peripheral and central tissue and ethanol perturbations of binding. Specific CRF binding sites were identified in adrenal, ventral prostate, spleen, liver, kidney, testis, and erythrocyte membranes. The addition of nanomolar concentrations of CRF induced a dose-related increase in rat adrenal membrane adenylate cyclase activity and in cyclic AMP levels in bovine chromaffin cells, which suggests that CRF may have an important regulatory role in various peripheral tissues. Similar to the effects of ethanol on BE binding, acute in vitro ethanol exposure produced a dose-related increase up to 0.2% and then a decline in CRF binding, whereas in vivo ethanol treatment decreased CRF binding to adrenal, testis, and erythrocyte membrane preparations. In addition, chronic ethanol exposure also lowered ^{125}I-rat CRF binding to pituitary membranes and reduced both basal and CRF-stimulated adenylate cyclase activity in pituitary membranes. Associated with lowered pituitary CRF binding and adenylate cyclase activity was a reduction in RNA levels.

Influence of ethanol on CRF receptors, adenylate cyclase activity, and POMC mRNA levels. Animals exposed continuously for 14 days to ethanol vapor in an inhalation chamber at sufficient ethanol vapor concentrations to maintain blood ethanol levels from 100 to 250 mg/100 mL exhibited approximately 36% lower CRF binding and 24% lower adenylate cyclase activity in anterior (AL) and neurointermediate lobe (NIL) membranes of the pituitary gland, as compared to nonethanol treated controls. Ethanol treatment for 1, 7, or 14 days produced a time-related decrease in POMC mRNA levels, relative to total RNA levels, in both the AL and NIL. Ethanol treatment caused a greater reduction in NIL POMC mRNA than AL POMC mRNA. Exposure to ethanol vapors for 14 days decreased immunoreactive beta-endorphin in plasma approximately 83%.

Development of a binding assay to assess the state of CRF and BE receptors in patients. Our demonstration that CRF and BE binding sites are present on erythrocyte (RBC's) membranes in both rats and humans and that binding changes of these peptides to RBC membranes parallel changes of classical CRF and BE target-organ binding, suggest that RBC-peptide binding assays could be used as an indirect method of assessing the state of CRF and BE receptors in a clinical setting. Further support for this notion was obtained when it was determined that a diurnal rhythm of CRF/BE binding to RBC's was observed in seven normal adult males, which paralleled the known diurnal rhythm of cortisol secretion.

Significance to Biomedical Research and the Program of the Institute:

Alcohol appears to exert its effects on CRF and BE binding sites in peripheral tissue membranes by fluidizing the membrane lipid bilayer. Furthermore, the demonstration of rCRF and BE binding sites in diverse peripheral tissue and high-affinity binding sites for CRF in rat adrenal medulla and bovine chromaffin cells and for BE in rat liver, plus the finding that activation of specific CRF binding sites in the adrenal gland and that of BE in liver stimulates the

adenylate cyclase/cAMP system, suggests that CRF and BE may have an important regulatory role in certain peripheral tissues. Consistent with our data that CRF may regulate some aspect of adrenal gland function is the recent finding of CRF-like activity in human adrenal glands. Although it is difficult to assign a specific function to the action of CRF in the adrenal gland at this time, CRF may modulate the release or biosynthesis of adrenomedullary catecholamines, pro-opiomelanocortin-related peptides, and/or enkephalin peptides. Further studies are needed to establish the precise role of CRF and BE in the physiological control of peripheral neuroendocrine function. Furthermore, alcohol also appears to exert its effects on pituitary CRF binding, basal and CRF-stimulated adenylate cyclase activity, and circulating immunoreactive BE levels. These ethanol-mediated alterations of the pituitary membranes, combined at least in part with a direct effect of ethanol on POMC gene transcription, may have resulted in decreased POMC mRNA levels and decreased circulating immunoreactive BE levels. Further studies on the effect of ethanol on the transcription rate of the POMC gene should provide additional insights into the mechanism of action of ethanol on the HPAA.

Proposed Course:

This project is continuing.

Publications:

Dave, J.R., Eiden, L.E., and Eskay, R.L.: Corticotropin-releasing factor binding to peripheral tissue and activation of the adenylate cyclase-adenosine 3',5'-monophosphate system. Endocrinology 116:2152-2159, 1985.

Dave, J.R., Eiden, L.E., Karanian, J.W., and Eskay, R.L.: Ethanol exposure decreases pituitary CRF binding, adenylate cyclase activity, pro-opiomelanocortin biosynthesis and plasma beta-endorphin levels in the rat. Endocrinology (in press).

Dave, J.R., Karanian, J.W., and Eskay, R.L.: Chronic ethanol treatment decreases specific non-opiate beta-endorphin binding to hepatic and kidney membranes and lowers plasma beta-endorphin in the rat. Alcoholism (in press).

Dave, J.R., Rubinstein, N., and Eskay, R.L.: Evidence that B-endorphin binds to specific receptors in rat peripheral tissue and stimulates the adenylate cyclase/cyclic AMP system. Endocrinology (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00245-02 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Effect of Ethanol on Protein Phosphorylation in AtT-20 Cells

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

PI:	R. Eskay	Research Physiologist	LCS, NIAAA
Others:	J. Bisserbe	Visiting Fellow	LCS, NIAAA
	K. Mishler	Biological Lab. Technician	LCS, NIAAA
	N. Rubinstein	Psychologist	LCS, NIAAA

COOPERATING UNITS *(if any)*

Biological Psychiatry Branch, NIMH (J. Patel)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.8	0.6	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The study of the effects of ethanol on protein phosphorylation in neurosecretory tissue will enhance our understanding of the deleterious impact of ethanol on biochemical events linking activation of membrane receptors to physiological responses such as hormone secretion. An endocrine cell line (AtT-20 cells) of anterior pituitary origin is used as a working model because it possesses multiple receptor types which regulate the synthesis and secretion of pro-opiomelanocortin (POMC) hormones (e.g., ACTH, alpha-MSH, beta-endorphin). The control of release and synthesis of POMC neuropeptides, as well as alterations in phosphorylated protein levels, is regulated in part by the adenylcyclase-cyclic adenosine monophosphate (AC/cyclic-AMP) system. Phosphorylated proteins obtained from broken-cell preparations or from intact cells are analyzed with single- and double-dimensional gel electrophoresis.

Our findings indicate that phosphorylation can be stimulated in broken-cell preparations by cAMP, calmodulin, and phorbol esters. Furthermore, forskolin, which activates the AC/cyclic-AMP system and enhances POMC hormone synthesis and release, also induces an increase in the nuclear and cytoplasmic protein kinases in intact cells. Following additional characterization of the system, the effect of ethanol on protein phosphorylation will be examined.

This project has been completed, with certain aspects being continued under a new project title.

PROJECT DESCRIPTION:

Investigators:

R. Eskay	Research Physiologist	LCS, NIAAA
J. Bisserbe	Visiting Fellow	LCS, NIAAA
K. Mishler	Biological Lab. Technician	LCS, NIAAA
N. Rubinstein	Psychologist	LCS, NIAAA
J. Patel	Visiting Associate	BPB, NIMH

Objectives:

The specific aims of this study are (1) to establish optimal assay conditions for the quantification of phosphorylated proteins in AtT-20 cells, (2) to determine the involvement of protein phosphorylation in membrane receptor and nonreceptor mediated changes in neuropeptide release, and (3) to explore ethanol-induced alterations in phosphorylated proteins that are linked to neuropeptide secretion.

Methods Employed:

Broken-cell phosphorylation preparation. Cultured AtT-20 cells are washed twice with phosphate-buffered saline (PBS), pH 7.4, and resuspended in either PBS or stopping buffer (50 mM tris phosphate, pH 7.4, 100 mM NaF, 10 mM EDTA, 5 mM EGTA, 5 mM DTT, 4 mg/mL leupeptin), which minimizes phosphatase activity. The cell suspensions are homogenized on ice with a teflon-glass homogenizer (eight strokes), and the homogenate is centrifuged for 10 minutes at 10,000 xg. The supernatant fluid is collected, the pellet is resuspended in an adequate volume of buffer, and an aliquot for protein determination is taken. For protein phosphorylation, an aliquot of membrane suspension (0.75 mg protein/mL) or supernatant fluid is incubated for 30 seconds in the presense of 5 μM [^{32}P]-ATP. The reaction is stopped by the addition of 50 μL of a solution containing 0.3% sodium dodecyl sulfate (SDS), 90 mM tris-HCL, 3% mercaptoethanol, and 15% glycerol and placed in a boiling water bath for 3 minutes. An aliquot of each heated sample is placed on a polyacrylamide gel electrophoresis apparatus.

Intact cell phosphorylation preparation. AtT-20 cells are washed twice in a phosphate-free buffer (Modified Kreb's Ringer [MKR] containing 132 mM NaCl, 4.8 mM KCl, 2.4 mM $MgCl_2$, 0.1 mM EGTA, 20 mM HEPES, 10 mM dextrose, and 1 mM $CaCl_2$). The cells are then incubated in MKR containing 100 μCi[^{32}P]/ml for 1 hour at 37° C. Cells are rinsed three times with DMEM and incubated for various periods of time with DMEM containing test drugs. At the end of the incubation interval, an aliquot of medium is removed for the determination of B-endorphin by RIA. The incubations are terminated by two rinses with DMEM followed by the addition of ice-cold stopping buffer containing 0.5% Triton-X. The lysed-cell preparation containing intact nuclei is centrifuged at 3500 xg for 5 minutes in order to separate nuclei from cytoplasmic material. The supernatant fluid is removed, the pellet is resuspended in stopping buffer, and each fraction is processed separately as follows: proteins are precipitated in 10% TCA, sedimented by centrifugation (12,000 xg for 70 minutes), and each sample is washed three times in a solution of diethylether/Triton X (1 vol:1 vol) to remove TCA and Triton X. Pellets are resuspended in 50 mM tris-phosphate buffer and dissolved in a solution

containing 90 mM tris-HCL, 3% SDS, 3% mercaptoethanol, and 15% gylcerol. Each sample is then placed in a boiling water bath for 5 minutes.

Separation of phosphorylated proteins is accomplished by SDS polyacrylamide (10% acrylamide, 0.74% bisacrylamide) slab electrophoresis. Ten to fifteen μ g of protein is applied per slot, and electrophoresis is performed until the tracking dye is 1-2 cm from the bottom of the gel. Gels are stained with Commassie Blue and destained by incubation in a solution consisting of 7% acetic acid, 5% methanol for 12 hours. Gels are dried, and autoradiography is performed with Kodak X-OMAT film. Developed autoradiograms are analyzed, using a Beckman spectrodensitometer. Relative molecular weights of [^{32}P]-proteins are determined by comparison with protein standards of known molecular weights.

Major Findings:

In the broken-cell phosphorylation procedure, we have found enhanced protein kinase activity in the presence of cAMP, calmodulin, and phorbol esters (activators of protein kinase C). Our results confirm previous findings in other cellular systems, which indicates that we have established optimal assay conditions for AtT-20 cells.

Slight modifications of standard procedures for protein phosphorylation in intact cells have enabled us to rapidly and reliably separate phosphorylated nuclear proteins from nonnuclear ones. Our optimized methods permit a high resolution and good separation of phosphorylated proteins of cytoplasmic and nuclear proteins, using one-dimensional gel electrophoresis. Forskolin, an adenylcyclase activator which enhances B-endorphin release, induced the increase of a 15,000 dalton cytoplasmic protein and two nuclear proteins (30,000 and 40,000 daltons).

Significance to Biomedical Research and the Program of the Institute:

We have established optimal assay conditions for the quantification and characterization of phosphorylated proteins in intact and broken-cell preparations of AtT-20 cells. This will enable us to evaluate the effect of ethanol in the presence of various stimulatory or inhibitory agents of neuropeptide secretion and protein phosphorylation.

Proposed Course:

This project has been completed, with certain aspects being continued under a new project entitled "PKC and the Secretion and Biosynthesis of Neuropeptides in AtT-20 Cells."

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00252-02 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

The Effect of Ethanol on Cyclic AMP and Beta-Endorphin Release from AtT-20 Cells

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

PI:	R. Eskay	Research Physiologist	LCS, NIAAA
Others:	K. Mishler	Biological Lab. Technician	LCS, NIAAA
	J. Dave	Visiting Associate	LCS, NIAAA
	J. Bisserbe	Visiting Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.2	0.8	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.)*

Ethanol perturbs a multitude of cellular functions because of its free access to all cellular compartments. The differential impact of ethanol on various tissues most likely relates to fundamental molecular differences between cells. In order to determine the concentration-dependent and temporal-dependent effect of ethanol on cellular events at the molecular level, we have been working with a well-characterized tumor cell line (AtT-20 cells) of anterior pituitary (AP) origin and dispersed AP cells, which contain multiple membrane receptors and secrete several stress hormones. Our approach is to understand the effect of ethanol on the sequence of events from membrane-receptor activation to intracellular-messenger systems to physiological responses (hormone release).

Our results suggest that ethanol differentially affects certain cellular events in a dose- and duration-related manner. Incubation of AP or AtT-20 cells with ethanol for 1-4 hours has only a marginal effect on hormone release and cAMP levels. In contrast, preincubation of cultured cells with ethanol for 24 hours alters cAMP-dependent hormone release, with only a slight effect on protein kinase C-mediated hormone release. Alteration of acute ethanol-induced membrane properties, as determined by CRF membrane binding, does not parallel the changes observed with corticotropin-releasing factor (CRF)-induced hormone release. Finally, ethanol treatment of cultured AP and AtT-20 cells results in a substantial reduction of POMC mRNA levels, suggesting that chronic ethanol treatment reduces the transcription of the POMC gene.

PROJECT DESCRIPTION:

Investigators:

R. Eskay	Research Physiologist	LCS, NIAAA
J. Dave	Visiting Associate	LCS, NIAAA
K. Mishler	Biological Lab. Technician	LCS, NIAAA
J. Bisserbe	Visiting Fellow	LCS, NIAAA

Objectives:

The ongoing aims of this group of studies 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 (neuropeptide release), and (2) to determine the concentration-dependent and time-dependent effects of ethanol on the various cellular events as outlined in (1).

Methods Employed:

A subclone of AtT-20/D16-16 cells, an anterior pituitary-derived tumor cell line, and dispersed anterior pituitary cells, which synthesize and secrete POMC-neuropeptides, were cultured. Prior to the 1-hour incubation of cells with various secretogogues with or without ethanol, the culture medium was removed and the cells were preincubated for up to 24 hours in the presence of ethanol. Following the 1-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 and pro-opiomelanocortin (POMC) messenger RNA (mRNA) in cultured cells was evaluated.

Major Findings:

Effect of ethanol on membrane-receptor activated BE and cAMP release from cultured AtT-20 and dispersed anterior pituitary (AP) cells. Incubation of AtT-20 and AP cells in ethanol (0.4 or 0.2%) for 1-4 hours followed by a 1-hour incubation in the presence of half-maximal stimulatory doses of isoproterenol (I) or CRF had no effect on I- or CRF-induced BE release or cAMP levels. Preincubation of AP and AtT-20 cells for 24 hours in 0.4% ethanol resulted in a lowering of CRF-induced BE secretion and cAMP levels.

Effect of ethanol on non-membrane-receptor-induced release of BE from cultured AtT-20 and AP cells. Incubation of AtT-20 cells in the presence of 0.4% and 0.2% ethanol for 4 or 24 hours significantly lowered forskolin (an apparent direct activator of adenyl cyclase)-induced release of BE. A reduction in cAMP levels paralleled the fall in BE secretion. Following 24 hours pretreatment, incubation of both AtT-20 and AP's with a half-maximal stimulatory dose of TPA, a phorbol ester which directly activates the protein kinase C system, resulted in a slight reduction in BE secretion as compared to TPA alone, without altering cAMP levels.

Effect of ethanol on [I^{125}]-CRF binding to intact AtT-20 cells and AtT-20 membranes. Intact AtT-20 cells and AtT-20 cell-derived membranes were incubated in the presence of ethanol concentrations ranging from 0.025-0.8% and [I^{125}]CRF. Maximal enhancement of specific CRF binding of 60-70% was achieved at 0.1% and 0.2% ethanol in membranes and intact cells, respectively. Enhanced CRF binding was reduced at concentrations in excess of 0.2% ethanol, but remained above controls (no ethanol) up to 0.8% ethanol.

Effect of ethanol on POMC mRNA levels in AtT-20, AP, and IL cells. Cells were preincubated for 24 hours with 0%, 0.2%, or 0.4% ethanol and basal release of B-endorphin (BE) was determined during a 1-hour release experiment. Incubation of AtT-20 and AP cells with 0.4% ethanol resulted in a consistent 25% reduction in basal release of BE. However, the content of BE did not change in either AP or AtT-20 cells following 24-hour incubation with 0.4% ethanol. Ethanol produced a dose- and time-dependent decrease in POMC mRNA levels in AtT-20 cells. Twenty-four-hour treatment of AtT-20 cells with 0.2%, 0.4%, or 0.6% ethanol produced approximately 0%, 60%, or 80% decrease in POMC mRNA levels, respectively. Treatment of AtT-20 cells with 0.4% ethanol for 8, 12, or 24 hours produced a 40%, 50%, or 60% decrease in POMC mRNA levels, respectively. Similarly, treatment of AP cells with 0.2%, 0.4%, or 0.6% ethanol for 24 hours produced a 40%, 50%, or 60% decrease in POMC mRNA levels, respectively. Treatment of primary cultures of IL cells with ethanol produced changes in mRNA levels which were similar in magnitude to those observed with AtT-20 cells.

Exploration of the mechanism of action of benzodiazepines and ethanol on BE and cAMP release. Ro54864, a 1-4 benzodiazepine, binds specifically to a peripheral type benzodiazepine receptor present in various tissues such as the kidneys, lungs, heart, testes, adrenals, pituitary, and brain. It has been suggested that Ro54864 might directly inhibit the calcium-calmodulin system. In AtT-20 cells, Ro54864 inhibits, in a dose-related manner (10^{-6} to 10^{-4} M), basal secretion and corticotropin releasing hormone- or forskolin-induced beta-endorphin (BE) release without significantly affecting cAMP levels. Furthermore, Ro54864 inhibits BE release induced by CGP 28392, a calcium channel activator, but does not inhibit BE release enhanced by A23187, a calcium ionophore. These results suggest that Ro54864 inhibits BE release by affecting the permeability of voltage-sensitive calcium channels and not by altering intracellular calcium mobilization. These results suggest that benzodiazepines might affect the release of BE by a direct mechanism of action on BE-secreting cells. The effect of ethanol on Ro54864, A23187, Ca^{++} mobilization, and flux are being evaluated in AtT-20 cells.

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:

These projects are continuing, and several manuscripts are in preparation.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00253-01 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Characterization and Regulation of Release of Atrial Natriuretic Peptides

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

PI:	R. Eskay	Research Physiologist	LCS, NIAAA
Others:	N. D'Souza	Visiting Fellow	LCS, NIAAA
	J. Dave	Visiting Associate	LCS, NIAAA
	K. Mishler	Biological Lab. Technician	LCS, NIAAA
	J. Karanian	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Clinical Studies, NIMH (N. Zamir, G. Skofitsch, D. Jacobowitz); Laboratory of Clinical Studies, NINCDS (Z. Zukowska-Grojec, M. Haass).

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.75	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.)*

The mammalian cardiac atria contain a family of peptides, collectively termed atrial natriuretic peptides (ANP's), which are derived from a common precursor and possess intrinsic natriuretic, diuretic, vasorelaxant, and endocrine effects. The availability of synthetic ANP's has enabled the development of radioimmunoassays (RIA's) to localize and characterize the various extraatrial ANP's. Sound methods have been developed for the determination of plasma ANP's in order to clarify which ANP's are physiologically important or released in vivo.

Recent immunocytochemical and RIA distribution studies have localized ANP's to areas of the CNS that suggest the ANP's are involved in central cardiovascular regulatory events and in the release and/or synthesis of certain pituitary gland hormones. Additional support for the possible involvement of ANP's in regulating pituitary gland function was obtained with the demonstration that high-affinity, specific ANP binding sites are present on pituitary gland membranes. Finally, two forms of ANP are present in plasma, and their release is enhanced from the atria by activation of atrial-stretch receptors, as well as adrenergic, cholinergic, and vasopressinergic agonists.

We are currently evaluating the effect of ethanol on the ANP system in animals and the possible involvement of ANP's in certain cardiovascular pathologies associated with alcohol-related disorders in patients.

PROJECT DESCRIPTION:

Investigators:

R. Eskay	Research Physiologist	LCS, NIAAA
N. D'Souza	Visiting Fellow	LCS, NIAAA
J. Dave	Visiting Associate	LCS, NIAAA
K. Mishler	Biological Lab. Technician	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA
N. Zamir	Visiting Associate	LCS, NIMH
Z. Zukowska-Grojec	Visiting Associate	LCS, NINCDS
M. Haass	Visiting Fellow	LCS, NINCDS
G. Skofitsch	Visiting Fellow	LCS, NIMH
D. Jacobowitz	Research Pharmacologist	LCS, NIMH

Objectives:

The specific aims of these studies are to determine the role of ANP's in the regulation of body fluid balance and blood pressure and to evaluate the potential effects of ethanol on the ANP system, which may be responsible in part for the cardiovascular pathology associated with alcoholism, such as hypertension and stroke.

Methods Employed:

Routine immunometric techniques were employed to localize and quantify ANP's in the CNS. Plasma levels of ANP's were determined by RIA, following established protocols for the extraction of peptides from plasma. All blood samples were obtained from conscious rats with indwelling arterial catheters following volume loading with either physiological saline, 5% glucose, or the administration of adrenaline, carbachol, or vasopressin. For the characterization of plasma ANP's, plasma extracts or synthetic ANP's were subjected to HPLC, utilizing a linear gradient of acetonitrile (ACN) from 25-55% ACN (Pump A, 0.1% TFA in water; Pump B, 0.1% TFA in ANC). One-mL fractions were collected over 60 minutes, dried down, and RIA'd.

Major Findings:

In conscious rats with chronic indwelling catheters, volume loading with isotonic saline or glucose enhanced circulating immunoreactive ANP's fourfold to fivefold, as determined by radioimmunoassay. Results obtained with the denervated-heart preparation indicate that neuronal influences are important in volume-loading induced release of ANP's. On the basis of high-pressure reversed-phase liquid chromatography of extracted plasma, the circulating, physiologically important ANP's in the rodent appear to be alpha-rat ANP_{5-28} (atriopeptin III) and alpha-rat ANP_{3-28} (ANF_{8-33}).

Furthermore, μg amounts of adrenaline or carbachol and ng amounts of vasopressin administered as a bolus-arterial injection resulted in a twofold to fivefold increase in circulating ANP's within minutes. Anesthetic doses of pentobarbital,

halothane, or ether also enhanced plasma ANP levels, whereas anesthetic doses of ethanol (5 gm/kg) had no effect on plasma ANP's.

Significance to Biomedical Research and the Program of the Institute:

Clearly, alteration of the purported role for ANP's in disorders of electrolyte balance and blood-pressure regulation could be a major factor in the development of cardiovascular disease and, therefore, merits intensive investigation. Since ethanol is known to alter vascular tonus and the ANP's are known to antagonize the pressor actions of certain substances (e.g., noradrenaline and angiotensin II), the possible effects of ethanol on ANP release or ANP sites of actions (e.g., kidney, vascular smooth muscle, and pituitary gland) need to be explored.

Proposed Course:

These studies are being continued, and four manuscripts are in preparation in addition to those in press or already submitted.

Publications:

Jacobowitz, D., Skofitsch, G., Keiser, H., Eskay, R., and Zamir, N.: Evidence for the existence of atrial natriuretic factor-containing neurons in the rat brain. Neuroendocrinology 40:92-94, 1985.

Samson, W.K., and Eskay, R.L.: Endocrine and neuroendocrine actions of cardiac peptides. In T. Moody (Ed.): Neural and endocrine peptides and receptors. New York, Plenum (in press).

Skofitsch, G., Zamir, N., Eskay, R., and Jacobowitz, D.: Distribution of atrial natriuretic factor-like immunoreactive neurons in the rat brain. Neuroscience (in press).

Zamir, N., Skofitsch, G., Eskay, R., and Jacobowitz, D.: Distribution of immunoreactive atrial natriuretic peptides in the central nervous system. Brain Research (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00254-01 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

PKC and the Secretion and Biosynthesis of Neuropeptides in AtT-20 Cells

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

PI:	R. Eskay	Research Physiologist	LCS, NIAAA
Others:	R. Parthasarathy	Visiting Associate	LCS, NIAAA
	K. Mishler	Biological Lab. Technician	LCS, NIAAA

COOPERATING UNITS *(if any)*

Biological Psychiatry Branch, NIMH (J. Patel)

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.5	1.3	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.)*

Enhanced protein phosphorylation is one of the intracellular events which invariably follow membrane receptor activation. Recently identified protein kinase C (also termed calcium- and phospholipid-dependent protein kinase) is a unique enzyme that phosphorylates specific proteins whose functions remain to be determined. Protein kinase C (PKC) requires a phospholipid and a diacylglycerol (DAG) for maximal activity. DAG is the hydrolytic product of membrane polyphosphoinositide breakdown, which occurs following membrane receptor occupancy, and is one of the initial events in signal transduction. The release of DAG during PI turnover substantially stimulates PKC activity over that observed in quiescent cells. The known co-carcinogen or tumor promotor TPA specifically increases membrane PKC activity. Associated with this increased membrane PKC activity is a reduction of cytosolic PKC activity, which suggests that the translocation of PKC is an important intracellular event.

Using a homogeneous population of cells (a mouse-derived, anterior pituitary tumor cell line [AtT-20s]) that respond to a variety of membrane and intracellular activators by releasing POMC-derived peptides, we have explored the involvement of PKC in hormone secretion by monitoring the release of beta-endorphin (BE) as well as membrane and cytosolic PKC activity. TPA treatment resulted in a dose-related increase in BE secretion and translocation of PKC from the cytosolic to the membrane fraction. A maximally stimulating concentration of TPA enhanced membrane PKC activity twofold within 1 minute and fivefold within 3 minutes. It would appear that activation (translocation) of PKC results in enhanced hormone release through a pathway that does not involve the adenylate cyclase/cAMP system.

PROJECT DESCRIPTION:

Investigators:

R. Eskay	Research Physiologist	LCS, NIAAA
R. Parthasarathy	Visiting Associate	LCS, NIAAA
K. Mishler	Biological Lab. Tech.	LCS, NIAAA
J. Patel	Visiting Associate	BPB, NIMH

Objectives:

The establishment of methods to determine the role of PKC in the secretion and biosynthesis of neuropeptides, using a homogeneous population of cells (AtT-20s) that secrete POMC-derived peptides. The evaluation of the effect of ethanol on PKC-dependent cellular events.

Methods Employed:

PKC was partially purified by DEAE ion exchange chromatography and assayed by the specific transfer of gamma [^{32}P]-ATP to the histone III fraction. Both cytosolic and Triton X-100 solubilized membrane fractions were used to monitor changes in PKC activity.

Major Findings:

Protein kinase C activity was studied in cytosolic and membrane fractions of AtT-20 cells. Cytosol obtained from quiescent cells transferred 182.9 picomole of [^{32}P]/min/mg protein, whereas Triton-solubilized membrane transferred 99.2 picomole/min/mg protein. Upon 15 minutes stimulation with 10^{-6} M TPA, the membrane fraction showed an activity of 359 picomole of [^{32}P]/min/mg protein. BE release, as determined by RIA, was enhanced threefold to fivefold in the presence of 10^{-6} M TPA.

Significance to Biomedical Research and the Program of the Institute:

The discovery of a new second-messenger system involving phosphoinositide turnover and the concomitant formation of IP_3 and DAG, which in turn mobilizes Ca^{++} and stimulates PKC activity, respectively, has provided a new system utilizing novel protein kinases and novel membrane-derived metabolites with second-messenger functions. Exploration of the involvement of the PKC system in cellular signal transduction will provide a better understanding of cellular regulatory events. Since ethanol is known to perturb both membrane and intracellular events, the potential impact of ethanol on the PKC system needs to be evaluated.

Proposed Course:

These studies will be continued to better understand the role of PKC in cellular events and to evaluate whether or not certain ethanol-induced changes in cellular function are mediated in part through the polyphosphoinositide-PKC system.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00259-01 LCS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Evaluation of Withdrawal Behavioral Changes in Rats Exposed to Ethanol Vapors

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

PI:	R. Lister	Visiting Fellow	LCS, NIAAA
Other:	J. Karanian	Senior Staff Fellow	LCS, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Clinical Studies

SECTION

Section of Neurochemistry

INSTITUTE AND LOCATION

NIAAA, 9000 Rockville Pike, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Rats that inhaled ethanol vapors for 7 or 14 days underwent a withdrawal syndrome following removal from the inhalation chambers. Approximately 60% of rats exposed to the vapors for 3 days also exhibited various withdrawal symptoms, but rats exposed to vapors for 24 hours showed no marked withdrawal reaction.

In a holeboard test performed approximately 11 hours after removal from the chambers, when blood alcohol concentrations were below the limits of detection, rats exposed to ethanol vapors for 24 hours or for 3, 7, or 14 days all had reduced exploratory and locomotor activities.

Whether or not this hypoactivity is a manifestation of a withdrawal reaction or reflects some other effect of exposure to ethanol vapors is currently under investigation.

PROJECT DESCRIPTION:

Investigators:

R. Lister	Visiting Fellow	LCS, NIAAA
J. Karanian	Senior Staff Fellow	LCS, NIAAA

Objectives:

To investigate the behavioral changes associated with withdrawal following various periods of exposure to ethanol vapors.

Methods Employed:

Male Sprague-Dawley rats are acclimated to ethanol inhalation chambers for 24 hours. Following the acclimation period, the vapor concentration in the chamber is increased to achieve blood ethanol levels between 150 and 250 mg/100 mL. Rats are exposed to this concentration for 24 hours, 3 days, 7 days, or 14 days. Control animals are similarly acclimated but are exposed to negligible concentrations of ethanol vapors for the duration of the experiment. After the exposure period, animals are removed from the chambers and their behavior is rated by an observer ignorant of the treatment conditions. The incidence of withdrawal symptoms (tail stiffening, tail and body tremors, extensor and body rigidity, and spontaneous seizures) are noted every 1-2 hours for a period of 30 hours.

Approximately 11 hours after removal from the chambers, after blood alcohol concentrations are below the limits of detection, the animals are tested individually in a holeboard apparatus for 10 minutes to assess their locomotor and exploratory activities.

Major Findings:

All animals exposed to ethanol vapors for 7 days and 14 days exhibited withdrawal reactions of varying intensity over the time period studied. Sixty percent of the animals exposed to the vapors for 3 days showed some degree of withdrawal reaction as assessed by observer ratings, but animals that inhaled the vapors for just 24 hours failed to exhibit observable withdrawal symptoms.

All groups of animals exposed to the ethanol vapors had reduced locomotor and exploratory head-dipping scores when tested in the holeboard apparatus.

Significance to Biomedical Research and the Program of the Institute:

The present results have two likely interpretations. The first is that ethanol vapors may produce changes such as eye irritation or damage to the olfactory system. If these changes persist after the animals have been removed from the chambers, reductions in locomotor activity and exploration would be anticipated. Should this be the case, caution should be exercised in attributing neurochemical or other changes resulting from ethanol exposure using this paradigm to elevated blood alcohol concentrations alone.

The second interpretation is that hypoactivity is a more sensitive measure of ethanol withdrawal than observer ratings and that a 24-hour exposure to ethanol vapors is sufficient to produce a mild withdrawal reaction. If this is the case, then it should be possible to screen compounds that might protect against withdrawal reactions, using the 24-hour rather than the 7-day exposure paradigm.

Proposed Course:

The next series of experiments will attempt to distinguish between the two interpretations offered above. The time course of the hypoactivity is being investigated, and other methods of administering ethanol are being used (e.g., i.v. infusions and i.p. injections).

Publications:

None

Annual Report of the Laboratory of Metabolism
National Institute on Alcohol Abuse and Alcoholism
October 1, 1984 to September 30, 1985
Richard L. Veech, M.D., D. Phil., Chief

Introduction

The work of the Laboratory of Metabolism can be divided into three broad areas:

1. Control of the rate of ethanol metabolism
2. Metabolic and pathological consequences of ethanol metabolism
3. Genetics of human alcoholism

In approaching the question of alcoholism, the laboratory consciously attempts to follow the classical medical approach toward any disease of unknown etiology. It therefore consciously restricts the number of problems it addresses concerning the wider spectrum of social abuses of alcohol. It is particularly concerned with that section of the population who, when they begin to drink, are unable to cease drinking on the following morning. It is also concerned with the effects of the metabolism of any amounts of ethanol, above those produced endogenously, and the consequences of such ingestion. This restriction of the scope of the social problems concerning the laboratory nevertheless require a wide view of pathophysiology, because of the ubiquitous nature of the metabolic effects of ethanol.

1. The Control of the Rate of Ethanol Metabolism

A. The Enzymes of Alcohol Metabolism

The control of the rate of ethanol metabolism is among the simplest of any commonly ingested substance. Its metabolism approaches a linear rate (Mellanby E, Alcohol: Its Absorption into and Disappearance from the Blood under Different Conditions. National Health Insurance, Medical Research Committee, Special Report Series 31, H.M.S.O., London, 1919) which may be approximately described by simple Michaelis-Menton kinetics (Lundquist F, Wolthers H. Acta Pharmacol Toxicol 14:265, 1958). It would, in fact, appear that the rate of ethanol metabolism may be adequately described both in vitro and in vivo by the kinetic constants of the first enzyme in its metabolic pathway, alcohol dehydrogenase (EC1.1.1.1). When those kinetic constants, which reflect the inherent properties of the enzyme itself, are solved in the equation which describes the steady-state rate for an ordered bi-bi reaction, an adequate description of the rate of ethanol metabolism results (Cornell NW, Crow KE, Leadbetter MG, Veech RL. Rate determining factors for ethanol oxidation in vivo and in isolated hepatocytes. In: Li TK, Schenker S, Lumeng L. eds. Alcohol and Nutrition. U.S. Government Printing Office, Washington D.C. pp 315-330, 1979).

From such simple yet apparently arcane observations a number of conclusions follow. The interesting and widely discussed variation in the rate of ethanol metabolism which exists between racial groups (Fenna D, Mix L, Schafer O, Gilbert JAL. Can Med Assoc J 105:472-475, 1971) or conversely the similarity of the rate of ethanol metabolism of closely related

individuals (Vessell ES, Page JG, Passananti GT. Clin Pharm Ther 12:192-201, 1971) is a function of the genetically determined structure of their one enzyme alcohol dehydrogenase. Unfortunately, 50 years of observations of the rate of ethanol metabolism in alcoholics versus normals clearly establishes that there is no significant difference in the rate of ethanol metabolism between alcoholics and nonalcoholics (Widmark EPM. K Fysion Sallsk Lund Forh 41:1, 1930; Mendelson JH, Stein S, Mello NK. Metabolism 14:1255-1256, 1965). The slight increase in the rate of ethanol clearance shown by many alcoholics can be adequately described by the hepatomegaly often accompanying that disease and the slight ability of ethanol to induce an increased amount of alcohol dehydrogenase in liver.

The deduction, based on simple kinetics, that the disease of human alcoholism does not involve abnormalities of the major enzymes of ethanol metabolism (e.g., alcohol dehydrogenase EC1.1.1.1 or aldehyde dehydrogenase EC1.2.1.3) has been borne out experimentally in surveys of liver biopsies of alcoholics where no difference from normals was found in the alcohol dehydrogenase or aldehyde dehydrogenase (Tipton KF, McCrodden JM, Weir DG, Ward K. Alcohol and Alcoholism 18:219-225, 1983). This lack of difference between normal and alcoholic individuals make the speculations (Bosron WF, Li TK, Vallee BL. Proc Nat'l Acad Sci U.S. 77:5784-5788, 1980; Jornvall H, Hempel J, Vallee BL, Bosron WF, Li TK. Proc Nat'l Acad Sci U.S. 81:3024-3028, 1984; Duester G, Hatfield GW, Bukler R, Hempel J, Jornvall H, Smith M. Proc Nat'l Acad Sci U.S. 81:4055-4059, 1984) that "the degree of susceptibility" of certain individuals to alcoholism, the fetal alcohol syndrome, or chlorpropamide alcohol flushing might be due to the variations in the isozymes of either alcohol dehydrogenase or aldehyde dehydrogenase seem unlikely.

Such simple kinetic considerations, when coupled with an analysis of the thermodynamics of the pathway of alcohol metabolism (Thacker SB, Veech RL, Vernon AA, Rutstein DD. Alcoholism: Clin Expt'l Res 8:375-383, 1984) make it equally unlikely that the reports that alcoholics (Korsten MA, Matsuzaki S, Feinman L, Leiber CS. N Eng J Med 192:386-389, 1975) or their relatives (Schuckit MA, Rayes V. Science 203:54-55, 1979) have elevated levels of blood acetaldehyde are correct. Again, when subjected to experimental tests, the siblings of alcoholics, given a challenge dose of ethanol, showed no difference in blood acetaldehyde level when greater care was taken in performing this most difficult analysis (Ward K, Weir DG, McCrodden JM, Tipton KF. IRCS Med Sci 11:950, 1983).

Further progress has been made in the continuation of the study of the intimate details of ethanol metabolism and the enzymes involved. A quantitative structure-activity analysis of 4- substituted pyrazoles was previously reported from this laboratory (Cornell NW, Hansch C, Kim, Henegar. Arch Biochem Biophys 227:81, 1983). It was pointed out then that pyrazoles, like perhaps all "specific inhibitors", affect more than one process when used in vivo or with whole cells. It had been shown for example (Evarts R. Biochem Pharmacol 31:1245-1249, 1982) that pyrazole, administered in vivo will induce a microsomal cytochrome P450, dimethylinitrosamine demethylase. It has subsequently been shown (Yang CS, Koop DR, Wang T, Coon MJ. BBRC in press, 1985; Koop DR, Crump BL, Nordblom GD, Coon MJ. Proc Nat'l Acad Sci U.S. 82 in press, 1985) that a diverse group of relatively polar agents such as ethanol, imidizole, trichlorethylene, acetone, pyrazole, isoniazid, and

similar agents all induce the same form of microsomal enzyme, the so-called cytochrome $P450_{LM3a}$ in Coon's nomenclature, which is identical with the nitrosomine demethylase activity and differs significantly from other monooxygenases. Our recently completed study of the capacity of 4-substituted pyrazoles to act as inducers and effectors of the activity of cytochrome P450s showed that this is solely dependent on the hydrophobicity of pyrazoles. Because of these relationships, it can reasonably be expected that pyrazoles will be strong inhibitors of $P450_{LM3a}$ activity. This is somewhat surprising since the proponents of the microsomal ethanol oxidizing system (MEOS) have defined that pathway as the pyrazole-insensitive component of hepatic ethanol metabolism. (Teschke R, Hasumura Y, Lieber CS. Arch Biochem Biophys 163:404-415, 1974).

Two papers were published in this area during the past year:
Sinclair J, Cornell NW, Zaitlin L, Hansch C. Induction of P-450 by alcohols and 4-substituted pyrazoles: comparison of structure activity relationships. Biochem Pharm (in press), 1985; and Hensch C, Klein T, McClarin J, Langridge R, Cornell NW. A QSAR and molecular graphics analysis of hydrophobic effects in the interactions of inhibitors of alcohol dehydrogenase. J Am Chem Soc (in press) 1985.

B. Compartmentation in the Control of Ethanol Metabolism

The initial steps of ethanol metabolism occur almost exclusively in liver where the initial reaction, catalyzed by alcohol dehydrogenase occurs in cytosol. The second step of ethanol metabolism, catalyzed by aldehyde dehydrogenase, occurs within the mitochondria and involves the low K_m aldehyde dehydrogenase. The acetate formed by the second reaction leaves the liver where it is subsequently metabolized in extra-hepatic tissue to carbon dioxide and water. The ionic and chemical environments of these two major tissue compartments are likely to differ in significant ways, but the precise nature of these differences is unknown. Formidable technical difficulties have long prevented the elucidation of the precise composition of the mitochondrial matrix. Nevertheless, it is clear that an understanding of the control of these first two steps of ethanol metabolism requires that the intimate details of the chemical composition of the mitochondrial and cytoplasmic compartments be achieved along with a thorough understanding of the laws governing these compositions under different physiological conditions.

This laboratory has been engaged in such studies since its inception. The mitochondrion is a dynamic compartment, changing minute to minute in both enzymatic and chemical composition, depending upon the metabolic and hormonal environment in which it is operating. Over the past year studies on the reversible enzymatic composition of the mitochondria induced by hormonal changes have been completed and published (Cornell NW, Janski AM, Rendon A. Compartmentation of Enzymes: ATP citrate lyase in hepatocytes from fed or fasted rats. Fed Proc 44:2448-2452, 1985).

In addition to the intracellular compartmentation, there is a growing body of evidence suggesting that, even within a relatively homogeneous organ such as liver, important differences are present between hepatocytes which reside in the periportal area as opposed to those in the area around the central vein. (Sasse D, Katz N, Jungerman K. FEBS Lett 57:83-88, 1975). During

the past year, a methodological advance in technique that may prove useful in examining such intrahepatic compartmentation has been published from this laboratory (Quistorff B, Grunnet N, Cornell NW. Digitonin perfusion of rat liver. Biochem J 226:289-297, 1985).

2. The Metabolic and Pathological Consequences of Ethanol Metabolism

Because the metabolism of ethanol affects the steady-state level not only of the NAD- redox state, but also the linked phosphorylation state or [ATP]/[ADP][Pi], it is quite understandable that ingestion of ethanol affects nearly all of the body's major metabolic pathways to a greater or lesser extent. Clearly, some of the effects of ethanol ingestion can be considered to be pathological, even in situations when the amount of ethanol ingested may be well within the limits generally considered safe in a particular society. What precisely is the safe limit of ethanol consumption, from a scientific and medical point of view, is not known. Rather strongly held opinions based on a variety of preconceptions, and now laws, govern the alcohol consumption of the bulk of the population, with the exception of the alcoholic who drinks presumably in response to some as yet undefined internal stimulus in spite of external norms.

From a scientific point of view, it now seems clear that any level of ethanol above that produced endogenously in the gut by the action of microorganisms can produce measurable effects. This is so because the physiological purpose served by the evolutionary development of the enzyme alcohol dehydrogenase was to remove endogenously produced ethanol almost completely on one pass through the liver (Krebs HA, Perkins JR. Biochem J 118:635, 1970). At what point the metabolic changes induced by the ingestion of ethanol become "pathological" is not known, nor is there evidence that it need be the same for all individuals. Thus the dose of ethanol doubling the relative risk for alcoholic cirrhosis was estimated to be 20 g per day for females, but 40 g per day for males (Pequignot G, et al. Int J Epidem 7:113-120, 1978). This study suggests that the dose of exogenous ethanol required to double the rate of cirrhosis in women is about 1 jigger of distilled spirits or 1 beer per day, which is about half the amount necessary for men. This level of ethanol approaches the estimated amounts of ethanol produced in the gut in humans and is an order of magnitude lower than the mean amount of 245 g per day estimated by Lelbach to be required to produce cirrhosis in humans (Lelbach WK. In: Research Advances in Alcohol and Drug Problems, Vol. 1, Gibbins RJ et al. eds. John Wiley & Sons, p. 124, 1974). The uncertainty as to "what level of alcohol consumption is good for you" is of course obscured not only by the economic, historic, religious, social, and political factors that bear on this problem, but on the inability to obtain accurate consumption data in humans over time, and on our lack of a thorough understanding of the metabolic consequences of ethanol metabolism. It is toward that latter goal that the program of this aspect of the Laboratory's work is directed.

A. Lipid Metabolism

The most well known of the medical complications of alcoholism is liver disease of several common types. These are: fatty infiltration, alcoholic hepatitis, alcoholic hepatitis and fibrosis, cirrhosis, and acute yellow atrophy. Fatty infiltration occurs in normals given acute exposure to

excessive alcohol over 2 to 3 days, alcoholic hepatitis includes both fatty and inflammatory changes, cirrhosis involves replacement of masses of hepatic tissue and its replacement with fibrous tissue, and acute yellow atrophy is an acute disease with fatty infiltration, inflammatory changes, and death of hepatic cells following consumption of up to one quart of distilled spirits per day for several months. Abnormalities of lipid metabolism are therefore central to the classic toxicities of alcohol ingestion.

In this area of work, two papers were published by the laboratory this year (Casazza JP, Veech RL. Quantitation of the rate of fatty acid synthesis. In: Storey J, ed. Lipid Research Methodology Alan R. Liss, New York, 1984, 231-240; King MT, Reiss PD. Separation and measurement of short chain coenzyme A compounds in rat liver by reversed phase high-performance liquid chromatography. Anal Biochem 146:173-179, 1985).

B. The Effects of Ethanol on Nitrogen Metabolism

Less well known but possibly more devastating are the effects of ethanol metabolism on the pathways of protein and nucleic acid metabolism. It is known both clinically and in folk wisdom that drinking is associated with exacerbation of gout. In addition to a redox state-induced decrease in the urinary excretion of uric acid by the kidney, the consumption of alcohol also increases the hepatic production of uric acid, the end product of purine metabolism. The precise mechanism of ethanol's effects upon this pathway of purine and pyrimidine synthesis, and their further conversion to DNA and RNA, was the subject of this study. One paper in this area appeared this year (Reiss PD, Zuurendonk PF, Veech RL. Measurement of tissue purine, pyrimidine, and other nucleotides by radial compression high-performance liquid chromatography. Anal Biochem 140:162-172, 1984).

C. Cerebral Glucose Utilization and Its Measurement

It is a widely held belief that since alcoholism involves a behavior, namely drinking excessive amounts of ethanol, the key to its understanding must lie in the brain. To the extent that any volitional act requires brain function, such a proposition is undeniable. However, there are by now numerous examples of stereotyped aberrant behaviors that have been understood as the result of a generalized metabolic defect. An example would be the self mutilatory behavior characteristic of Lesch-Nyhan syndrome which results from a generalized deficiency of the enzyme of purine metabolism, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8). How such an enzyme defect with its resultant metabolic abnormalities expresses itself in a stereotyped abnormal behavior is not known. A similar situation is possible in the case of alcoholism. That is, in susceptible individuals, the metabolic consequences secondary to consuming alcohol may result in the stereotyped behavior of excessive drinking which is characteristic of that disease.

While ethanol itself is not metabolized by the brain, its metabolic products acetaldehyde and acetate are. In addition, peculiar compounds such as acetoin can be produced by the brain following ethanol administration (Veech et al. Curr Topics in Cell Reg 18:151-179, 1981) thus robbing the brain of the energy it would normally derive from the metabolism of glucose, its major fuel. Recently there has been an increased interest in using various radioactive derivatives of glucose to measure the "metabolic rate" of

various regions of the brain under different metabolic or pathological conditions. The most popular of these techniques has been the method of using deoxyglucose (Sokoloff L et al. J Neurochem 28:897, 1977) or its positron-emitting analogue as a measure of cerebral energy metabolism. Numerous papers using this technique have recently appeared, including studies of alcohol withdrawal.

It is now clear, however, that the assumption underlying this method, namely that the glucose entering the brain over 45 minutes goes almost entirely to pyruvate and hence through the Krebs cycle to carbon dioxide and energy via the electron transport system is not correct (Huang M-T, Veech RL. J Biol Chem 257:11358-11363, 1985). The precise degree to which the cerebral glucose is being phosphorylated in brain to glucose 6-phosphate and then being dephosphorylated to an as yet unknown compound (Nelson T, Lucignani G, Atlas S, Crane AM, Dienel GA, Sokoloff L. Science 229:60-62, 1985) is not yet known. To the extent that this unknown compound accumulates in the brain to emit radioactivity either from a ^{14}C label on deoxyglucose or from a fluorine label during positron emission spectroscopy in patients undergoing PET scanning for evaluation of cerebral function, such a signal represents an erroneous estimation of cerebral energy demands. This is because this dephosphorylated glucose derivative is out of the main glycolytic pathway and hence out of the major cerebral energy-producing pathway. The nature of the reaction producing the dephosphorylation of glucose 6-phosphate also remains to be determined. One paper has been published in this area from the laboratory this year (Huang M-T, Veech RL. Metabolic fluxes between [^{14}C]-2-deoxy-d-glucose and [^{14}C]-2-deoxy-d-glucose 6-phosphate in brain in vivo. J Neurochem 44:567-573, 1985).

D. The Effects of the Metabolic Products of Ethanol on Inorganic Pyrophosphate Metabolism

A great deal of the alcoholism literature has focused on the possible effects of acetaldehyde (1) forming condensation products with neurotransmitters (Davis VE, Walsh MJ. Science 167:1005-1006, 1970; (2) causing the formation of opiate or encephalin type molecules in the brain, which in turn cause the exposed animal to consume more alcohol thus forming the biochemical link between alcoholism and "substance abuse" (Myers RD, Melchior CL. Science 196:554-556, 1977); and (3) being a genetic marker for alcoholism (Schuckit MA, Rayes V. Science 203:54-55, 1979). For the kinetic and thermodynamic reasons we have discussed previously (Thacker SB, Veech RL, Vernon AA, Rutstein DD. Alcoholism: Clin and Expt'l Res 8:375-383, 1984), such hypotheses seem unlikely for the simple reason that acetaldehyde levels are kept extremely low by virtue of the kinetics and the thermodynamics of the reactions in which this molecule is involved in the animal body.

In contrast to the attention paid to acetaldehyde, relatively little attention has been paid to the effects of the major product of hepatic ethanol metabolism, acetate (Lundsgaard E. C.R. Lab Carlsberg Ser Chim 22:333-337, 1938; Lundguist F, Tygstrup N, Winkler K, Mellengaard K, Munck-Peterson S. J Clin Invest 41:955-961, 1962). It turns out that the metabolism of acetate has multiple effects upon the major metabolic pathways of the body such as gluconeogenisis, purine and pyrimidine synthesis, DNA and RNA metabolism, genetic expression, and mineral metabolism particularly Ca^{2+}, phosphate, and inorganic pyrophosphate. In addition to the role CA^{2+} plays as an

intracellular regulator, which is too complex to discuss here, acetate's effects are likely to play a role in the little studied but painful and disabling effects of osteoporosis, which effects not only the elderly female population, but to a greater extent alcoholics.

In an interesting adventitious finding from our further studies, the 2 to 4 mM inorganic pyrophosphate the accumulates in the mitochondrial matrix during acetate metabolism was found to be NMR silent even though this amount was well within the sensitivity of the instrument to detect. This finding therefore has far reaching implications for the understanding of the fundamental nature of the environment within the mitochondrial matrix.

One paper has been published in this area during the past year (Veech RL, Gitomer WL, King MT, Balaban RS, Costa JL, Eanes ED. The effect of short chain fatty acid administration on hepatic glucose, phosphate, magnesium and calcium metabolism. In: Brautbar N, ed. Myocardial and Cellular Bioenergetics and Compartmentation (in press) 1985).

E. Regulation of Oxygen Consumption by Hormones and the Interaction of Ethanol with these Regulatory Properties

A long and widely held view of both the cerebral and hepatic toxicity of ethanol is that chronic ingestion of ethanol leads to an increase in the measured activity of the Na^+/K^+ ATPase (EC 3.6.1.3) in brain (Israel Y, Kalant H, LeBlanc E, Bernstein J, Salazar I. J Pharmacol Exp Ther 174:330-336, 1970) and in liver (Burnstein J, Videla L, Israel Y. Biochem J 134:515-521, 1974). It was held that "after chronic administration of ethanol, the sodium pump becomes the pacemaker of cellular metabolism" (Bernstein J, Videla L, Israel Y. Ann NY Acad Sci 242:560-572, 1974). At about the same time it was reported that thyroid hormone administration also led to an increase in the measured activity of the Na^+/K^+ ATPase activity, accounting for the calorigenic effects of thyroid hormone. The increased oxygen consumption observed after adrenergic stimulation was attributed to a similar mechanism and a unifying mechanism was postulated that chronic ethanol administration led to complex changes in the plasma membrane of cells, which in turn effected the activity of the Na^+/K^+ ATPase and other membrane transport processes (Bernstein J, Videla L, Israel Y. Ann NY Acad Sci 242:560-572, 1974).

This general theory continues to guide a number of projects in alcohol research to this day. More pertinent to patient care however, this theory led to the proposal that potentially lethal complications such as alcoholic hepatitis, with an approximately 60 percent mortality, could be successfully treated with the antithyroid drug propylthiouracil (Orrego H, Kalant H, Israel Y et al. Gastroenterology 76:105-115, 1979). In spite of double-blind studies that failed to find significant benefit from the use of propylthiouracil in alcoholic hepatitis (Halle P, Pare P, Kaptein E, Reynolds T. Gastroenterology 82:925-931, 1982) interest in this area persists and clinical trials continue.

As a general proposition, the work of this laboratory over the past 5 years gives no support to the theory that alcohol exposure leads to a hypermetabolic state either in liver or in the whole organism (Schaffer WT, Denckla WD, Veech RL. Alcoholism: Clin Exptl Res 5:192-197, 1981). While there

can be no doubt that hormones such as thyroxine and the catechol amines can increase the rate of tissue oxygen consumption, the mechanism whereby this occurs is complex and not due simply to a unitary action upon the Na^+/K^+ ATPase which acts upon cellular metabolism as a master pacemaker. In our view, Na^+/K^+ ATPase is in fact an enzyme catalyzing a near equilibrium state between the extracellular and intracellular water, the electrolytes Na^+ and K^+, and an energy term reflecting the differences between the extracellular cationic Na^+ Donnan forces and the intracellular anionic Donnan-active material.

Furthermore, a number of factors appear to regulate the tissue oxygen consumption, as might be expected. Among the less well known hormones that appear to possess this property is growth hormone, which seems to have the property of inhibiting some of the cellular actions of thyroid hormone. There is an extensive literature on the interactions of growth hormone in relation to alcohol consumption and alcoholic liver disease. (For review see Morgan MB. Brit Med Bull 38:35-42, 1982.)

Three papers covering this area of the work of the laboratory have appeared this year (Schaffer WT. Effects of growth hormone on lipogenic enzyme activities in cultured hepatocytes. Am J Physiol 248:E719-E725, 1985; Schaffer WT, Mehlman MT, Tobin RB, Veech RL. Selective inhibition of the effects of thyroid hormone on rat liver by 5,5' diphenylthiohydantoin. J Toxicology and Industrial Health (in press) 1985; Veech RL. Pyridine nucleotides and the control of metabolic processes. In Dolphin D, Puolson R, Aoramovic O, eds. Co-enzymes and Co-factors: Pyridine Nucleotides John Wiley & Son, New York (in press), 1985).

3. The Genetics of Human Alcoholism

It is now generally accepted that there is a genetic component in severe alcoholism in males (Bohman M. Arch Gen Psychiatry 35:269-276, 1978) resulting in a threefold increase in the incidence of alcoholism in the male offspring of alcoholics even if raised apart from the alcoholic parent. Thus Bohman's study for the first time clearly showed the difference between the disease of alcoholism and a socially produced form of aberrant behavior such as criminality. Bohman's studies with adoptees showed that while criminality could be learned from the parents, alcoholism has a definite genetic component.

Nevertheless, there has been little progress since that time in elucidating the mechanism whereby this genetic propensity expresses itself. Many learned articles discussing the polygenic, multifactorial, isozymic, biopsychosocial, neuropharmacological possibilities of exploring the last frontier of the brain do not change the fact that our techniques for determining how genetic diseases express themselves is still very primitive. This is particularly so in diseases involving the brain, because of the complexity of that organ. Many less complex diseases that are clearly genetic such as cystic fibrosis (the most common lethal genetic disease in the United States), diabetes, or manic depressive disease remain without a definition of their primary defect or gene product involved. It should therefore not be either a source of embarrassment or something to be denied, if this aspect of alcoholism research remains in a primitive state so soon

after the general recognition of the genetic component existing in alcoholism (which was, after all, previously considered either a moral weakness or a psychological aberration). It is likely that, given the primitive nature of our knowledge, much more detailed biochemical work elucidating the intimate details of the biochemical changes in alcoholics, both before and after the development of the clinical manifestations of their disease, will be required before a central gene product defect can be identified.

If no "breakthroughs" have occurred since the publication of the Bohman paper, some progress has nonetheless been made. First, the detailed study of the kinetics and thermodynamics of the major pathway of alcohol metabolism makes it unlikely that any of those enzymes are defective in the alcoholic (see section 1.) This prediction has been confirmed by published studies of the isozymes of both alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase (EC 1.2.1.3) from the Medical Research Council Human Genetics Unit and Trinity College showing that there is no correlation between these enzymes and the disease of human alcoholism (See Ricciardi BR, Saunders JB, Williams R, Hopkinson DA. Pharm Biochem & Behav 18:61-65, 1983; Tipton KF, McCrodden JM, Weir DG, Ward K. Alcohol & Alcoholism 18:219-225, 1983).

The survey of neurotransmitters in alcoholics has yet to produce significant findings. Because of the pervasive nature of the action of alcohol it may be predicted that nearly all neurotransmitters will change either up or down during administration of alcohol or during its withdrawal. Just as certainly, however, the minimal nature of the defect in the alcoholic or potential alcoholic make it unlikely that a defect which will undoubtedly be found in the major neurotransmitter systems will be cause and not effect.

In considering this problem our approach has been to consider apparent differences in blood metabolites of a minor metabolic pathway in alcoholics and nonalcoholics consuming ethanol (Felver ME, Lakshmanan MR, Wolf S, Veech RL. In: Thurman RG ed. Alcohol and Aldehyde Metabolizing Systems IV. Plenum Press, New York, 1980:229-235; Rutstein DD, Nickerson RJ, Vernon AA, Kishore P, Veech RL, Felver ME, Needham LL, Thacker SB. Lancet ii:534-537, 1983). The existence of these pathways in humans was unknown before the beginning of this work (Veech RL, Felver ME, Lakshmanan MR, Huang M-T, Wolf S. Curr Topics in Cell Reg 18: 151-179, 1981; Casazza JP, Felver ME, Veech RL. J Biol Chem 259: 231-236, 1984); however, their existence had been suspected by earlier biochemical and microbiological work and by the existence of these otherwise inexplicable diols, 1,2-propandiol and 2,3-butandiol, in the blood of alcoholics. We continue to examine the enzymatic basis for the metabolism of these compounds, to develop methods for their assay, to determine their organs of origin and their ultimate metabolic fate, the limits of their specificity for alcoholism, and whether these pathways are primarily induced and the extent to which that induction is genetically determined.

Two papers have been published in this area so far this year (Casazza JP, Fu JL. The measurement of acetol in serum. Anal Biochem (in press), 1985; Casazza JP, Ishii H, Veech RL. The measurement of 2,3-butandiol and 1,2-propandiol in "flushing" and "non-flushing" Japanese. In: Meyers RD. ed. Alcohol 3:401-404, 1985).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00019-07 LM

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pyrazoles as Affectors of Alcohol Dehydrogenase In Vitro and In Vivo

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: N. Cornell Research Chemist LM, NIAAA

COOPERATING UNITS *(if any)*
Biochemistry Department, Dartmouth University, Hanover, NH (J. Sinclair)

LAB/BRANCH
Laboratory of Metabolism

SECTION
N/A

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.25	0.25	

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

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

As a means of comparing the functional properties of an enzyme in dilute solution in vitro with those for the same enzyme acting in its normal cellular environment, a study was conducted with 4-substituted pyrazoles as inhibitors of rat liver alcohol dehydrogenase in vitro and ethanol oxidation in isolated rat hepatocytes. Inhibitor constants (K's) for the same set of pyrazole derivatives were also determined for human liver alcohol dehydrogenase. The best fitting equations were derived to relate the K's to the chemical nature of substituents. These quantitative structure-activity relationships show that pyrazoles with stronger electron-withdrawing substituents are weaker inhibitors both for the enzyme in vitro and, to an equal extent, for ethanol oxidation by intact cells. Inhibitor effectiveness is also dependent on substituent hydrophobicity, but, while increasing hydrophobicity makes stronger inhibitors of the enzyme in vitro, it can diminish the effectiveness in vivo by decreasing permeability through the cell membrane. A structure-activity analysis of published K's for pyrazoles acting against human -ADH indicates that its active site differs from those in other alcohol dehydrogenases. Pyrazoles are also inducers of microsomal cytochromes P-450, and a structure-activity analysis of this phenomenon shows that it depends entirely on the hydrophobicity of the pyrazoles, with electronic and steric factors making little or no contribution.

PROJECT DESCRIPTION:

Investigators:

N. Cornell	Research Chemist	LM, NIAAA
J. Sinclair	Assistant Professor	Dartmouth Univ.

Objectives:

The long-range objective of this project is to accurately describe the behavior of alcohol dehydrogenase in vivo, and to define the factors that determine the rate of ethanol elimination in vivo.

Methods Employed:

The first phase of the work involved a quantitative structure-activity analysis for pyrazoles acting as inhibitors of alcohol dehydrogenase in vitro and of ethanol metabolism by intact cells. The second phase involves a similar analysis for the induction by pyrazoles of cytochromes P-450 in cultured hepatocytes. The same pyrazoles tested as inducers have also been characterized with regard to their ability to inhibit mixed function oxidation by microsomes in vitro. The activity used as the test system is hydroxylation of biphenyl to 4-hydroxybiphenyl, which can be measured fluorometrically in alkaline solutions. It will be interesting to see what the correlation is between the effectiveness of pyrazoles as inducers and as inhibitors of these microsomal activities.

Major Findings:

In the first phase, a study was conducted with 4-substituted pyrazoles as inhibitors of rat liver alcohol dehydrogenase in vitro and ethanol oxidation in isolated rat hepatocytes. Inhibitor constants (Ki's) for the same set of pyrazole derivatives were also determined for human liver alcohol dehydrogenase. The best fitting equations were derived to relate the Ki's to the chemical nature of substituents, and these quantitative structure-activity relationships show that the effectiveness of a 4-substituted pyrazole is a function both of the hydrophobicity and the electron-withdrawing properties (Hammet's meta) of the substituent. The coefficients of meta (Hammet's) were -2.06 with the human and -1.80 for isolated rat liver cells; the value was also -1.80 for the rat enzyme in vitro. The inhibitory effectiveness was also strongly dependent on the hydrophobicity of the substituent placed on pyrazole. With the rat and human enzymes in vitro, this relationship was linear, while with intact cells, where the inhibitor must pass through the cell membrane before interacting with the enzyme, the dependence on hydrophobicity was parabolic. Thus, while increasing substituent hydrophobicity makes for stronger inhibitors of alcohol dehydrogenase in vitro, it may, by decreasing the access to the enzyme, diminish the effectiveness in vivo. A quantitative structure-activity analysis was also performed on published data for the inhibition by 4-substituted pyrazoles of -ADH, a minor form of human alcohol dehydrogenase that has unusual kinetic properties. In contrast to the results with the alcohol dehydrogenases used in our experiments, the Hammet value was +3.53 for pyrazoles acting against -ADH; i.e., electron-withdrawal by the substituent makes, in this instance, a more potent inhibitor. This supports electrophoretic and immunochemical indications that -ADH and the major human alcohol dehydrogenases are products of different genetic systems.

In the second phase, pyrazole and five 4-substituted derivatives have been tested, and the results indicate that induction is primarily dependent on the hydrophobicity of the substituent. The 4.5-fold induction by 4-pentylpyrazole is equivalent to that obtained with an optimum dose of propylisopropylacetamide (PIA) which, previously, was the best of the known inducers of P-450s. The iodo-, methoxy-, cyano-, and acetyamino-derivatives have been tested, and it has been possible to quantitatively define the substituent parameters that influence the actions of pyrazoles as inducers of cytochromes P-450. In this instance, the best fitting equation relating activity as inducers to the chemical nature of the pyrazoles was one with only a single term for hydrophobicity ($r = 0.95$), and the addition of electronic and steric terms did not improve the fit. These results have some implications for the mechanism of induction of the barbiturate-type P-450s, i.e., that the inducers act by binding to the cytochrome embedded in the hydrophobic, lipid-rich microsomal membrane.

Significance to Biomedical Research and the Program of the Institute:

This work shows that the major factor determining the rate of ethanol elimination in vivo is the cellular activity of alcohol dehydrogenase. It confirms in a unique way that this enzyme behaves in the same way in the test tube as it does in its natural environment, the cell. The results also provide, for the first time, some quantitative criteria for designing inhibitors to block ethanol and methanol toxicity in vivo. It also gives insight into the mechanism of induction of cytochromes P-450, information that has not been forthcoming from other approaches to this problem.

Proposed Course:

This project will continue in FY 1986.

Publications:

Hansch, C., Klein, T., McClarin, J., Langridge, R., and Cornell, N.W.: A QSAR and molecular graphics analysis of hydrophobic effects in the interactions of inhibitors with alcohol dehydrogenase. J. Med. Chem. (in press).

Sinclair, J., Cornell, N.W., Zaitlin, L., and Hansch, C.: Induction of cytochrome P-450 by alcohols and 4-substituted pyrazoles: Comparison of structure-activity relationships. Biochem. Pharmacol. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00026-03 LM

PERIOD COVERED
October 1, 1984 to September 30, 1985

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)*
PI: N. Cornell Research Chemist LM, NIAAA

COOPERATING UNITS *(if any)*
Department of Biochemistry
University of Copenhagen, Denmark (N. Grunnet)

LAB/BRANCH
Laboratory of Metabolism

SECTION
N/A

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

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
0.75	0.25	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 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 μM 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.

PROJECT DESCRIPTION:

Investigators:

N. Cornell	Research Chemist	LM, NIAAA
N. Grunnet	Associate Professor	Univ. Copenhagen

Objectives:

In current discussions, the term metabolic regulation generally implies phenomena such as allosteric inhibition or activation, enzyme interconversion, and induction or repression. Perhaps because it is so fundamental, it often is forgotten that enzyme compartmentation plays a major role in the integration and control of cellular metabolism. One example is the production within mitochondria of the carbamylphosphate used to initiate ureogenesis and the production in the cytosol of the carbamylphosphate for the biosynthesis of pyrimidines. Thus, having two CP synthetases located in different cellular compartments prevents the process of ammonia elimination from interfering with pyrimidine biosynthesis. Cellular compartmentation of proteins takes on added significance in view of recent discoveries of receptor recycling between the plasma membrane and the cell interior, and of maturation and translocation into organelles of proteins synthesized in the cytosol under control of the nuclear genome. These considerations make it desirable to be able to rapidly and accurately quantitate the subcellular distribution of enzymes and other macromolecules.

Methods Employed:

The problems outlined above have been studied in the present work with the use of digitonin, a nonionic, weak detergent that forms a tight complex with cholesterol. Digitonin had been found in the 1960's by Levy and Schnaitman and Greenawalt to be useful in selectively releasing enzymes from various submitochondrial compartments. Vignais in 1971 extended that observation to show that the rate at which digitonin would disrupt cellular membranes depends on the cholesterol content (e.g., plasma membrane; outer mitochondrial and microsomal membranes; inner mitochondrial membranes). An important application of those developments occurred in 1974 when Zuurendonk and Tager used digitonin to fractionate isolated hepatocytes in order to study metabolite compartmentation. The procedure described by those workers and applied by others required a 30- to 60-second fractionation period, and, as we believed those times to be long relative to many cellular processes, in 1980 we undertook a systematic evaluation of the digitonin fractionation of isolated rat hepatocytes.

Major Findings:

(1) The establishment of methods that permit in 3 to 10 seconds the fractionation of rat hepatocytes into cytosolic and particulate materials;

(2) Demonstration that the method provides a means of quantitating subcellular enzyme compartmentation that is much more rapid and accurate than traditional techniques of homogenization and differential centrifugation;

(3) The discovery that, although 15 enzymes behave as predicted from results with classical techniques, three others (aspartate aminotransferase, malate dehydrogenase, and ATP citrate lyase) have quite unexpected distributions;

(4) The discovery of a fraction of ATP citrate lyase that changes subcellular location in response to the metabolic state of the cell, which is phosphorylated in response to glucagon, and for which the degree of phosphorylation depends on the subcellular location.

The validity of the results listed in (3) above was established by electrophoresis showing that the cytosolic fractions obtained by digitonin treatment of hepatocytes contain none of the mitochondrial isozymes of aspartate aminotransferase or malate dehydrogenase or vice versa. Thus, the cytosolic: mitochondrial distributions of those enzymes are 22:78 for MDH as opposed to literature values of 70:30; for AspAT the corresponding values are 15:85 (digitonin) and 50:50 (literature). The digitonin procedure also indicates that adenylate kinase is located exclusively in the mitochondrial intermembrane space in contrast to published conclusions that this enzyme is 20% cytosolic.

Although digitonin fractionation has been extensively used to study metabolites, we have been less interested in that application because this or any other cell compartmentation fractionation procedure gives only total contents while the values needed are concentrations of free, metabolically active substrates. We have, however, studied this application to establish that, for obtaining valid measurements of adenine nucleotide compartmentation, the exposure to digitonin must not exceed 7 seconds with the fractionation medium at 1° C. These limitations are much narrower than those accepted by any of the groups that have reported metabolite compartmentation values obtained by the digitonin technique.

During this year, a major discovery was that the intact liver could be rapidly fractionated by perfusing it with digitonin. Normal perfusion medium (Krebs/Henseleit saline) contains calcium ions, and it was found that those ions cause disruption of the mitochondrial inner membrane. Deletion of calcium ions permits selective release of cytosolic materials with little release of enzymes in the mitochondrial matrix. In addition to effecting a subcellular fractionation, digitonin perfusion of the liver also permits a selective fractionation (by simply altering the direction of perfusion) of cells in the periportal and perivenous regions.

Significance to Biomedical Research and the Program of the Institute:

Alcohol exerts its effects on hepatic metabolism by altering the redox state of the pyridine nucleotides, and a full understanding of those effects requires reliable quantitative information about enzymes such as malate dehydrogenase and aspartate aminotransferase that are setting the redox state, and enzymes like those of lipogenesis that respond to the redox state.

Proposed Course:

This project will continue in FY 1986.

Publications:

Cornell, N.W., Janski, A.M., and Rendon, A.: Compartmentation of enzymes: ATP citrate lyase in hepatocytes from fed or fasted rats. Federation Proceedings 44:2448-2452, 1985.

Quistorff, B., Grunnet, N., and Cornell, N.W.: Digitonin perfusion of rat liver. Biochem. J. 226:289-297, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 00027-03 LM

PERIOD COVERED
October 1, 1984 to September 30, 1985

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)*

PI: N. Cornell	Research Chemist	LM, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Metabolism

SECTION
N/A

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.75	0.25	0.50

CHECK APPROPRIATE BOX(ES)

☐ (a) 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.

PROJECT DESCRIPTION:

Investigators:

N. Cornell Research Chemist LM, NIAAA

Objectives:

The specific aims of this project are to establish conditions that permit the induction of aminolevulinic acid synthase to be studied in suspensions of isolated hepatocytes; to analyze the kinetics of messenger RNA transcription and translation and the movement of newly synthesized aminolevulinic acid synthase from cytosol to mitochondria; and to define the mechanism by which alcohol acts to increase the activity of the enzyme.

Methods Employed:

Hepatocytes are prepared from rat liver by the collagenase perfusion technique using a medium supplemented with a plasma amino acid mixture. Most amino acids are present at the concentrations characteristic of rat blood, but six of the essential amino acids shown to have strong activity in suppressing protein degradation are present at 4x plasma concentrations. The cells are also isolated in the presence of penicillin and streptomycin to permit their long-term retention in suspension without bacterial overgrowth. Cells from normal and induced animals are then subjected to digitonin fractionation, and the subcellular distribution of aminolevulinic acid synthase is measured relative to the marker enzymes, lactate dehydrogenase (cytosolic) and citrate synthase (mitochondrial).

Major Findings:

As this project is in its early stages, the major results to date have been methodological. A thorough evaluation has been conducted of the alternative methods of assaying synthase activity, and a radiochemical procedure has been developed that has the sensitivity and specificity required for working with small amounts of biological material. It also has been found that perfusion of the liver with the 4x plasma amino acid mixture permits retention of induced levels of aminolevulinic acid synthase in isolated hepatocytes, and initial fractionations have shown that the enzyme activity is 40% cytosolic and 60% mitochondrial. It has been found that the rat liver enzyme is bound by Cibacron Blue-Sepharose, and use of that affinity ligand should aid in purifying the cytosolic enzyme so that antibodies can be raised to it. The antibodies will then be used to precipitate polysomes containing the mRNA for aminolevulinic acid synthase.

Significance to Biomedical Research and the Program of the Institute:

The general problem of protein movement between various subcellular compartments is relevant to many areas of biomedical research, and the approach to that problem described here should lead to more rapid and reliable measurements of protein movement than can be obtained by classical techniques. This work will also provide insight into the means by which alcohol consumption alters heme biosynthesis and metabolism.

Proposed Course:

This project will continue in FY 1986.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00034-01 LM

PERIOD COVERED
October 1, 1984 to September 30, 1985

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)*

PI:	J. Casazza	Senior Staff Fellow	LM, NIAAA
Others:	R. Veech	Chief	LM, NIAAA
	W. Schaffer	Senior Staff Fellow	LM, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Metabolism

SECTION
N/A

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, MD 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.

PROJECT DESCRIPTION:

Investigators:

J. Casazza	Senior Staff Fellow	LM, NIAAA
R. Veech	Chief	LM, NIAAA
W. Schaffer	Senior Staff Fellow	LM, NIAAA

Objectives:

The purpose of this study was to determine if under any conditions the tissue content of glycolytic and pentose cycle intermediates were interdependent. It has been proposed that flux through glycolysis and the level of nucleotide synthesis is dependent on the level of 6-phosphogluconate (Smith, S.B., and Freedland, R.A.: J. Biol. Chem. 254:10644-10648, 1980; Sommercorn, J., Steward, T., and Freedland, R.A.: Arch. Biochem. Biophys. 232:579-584, 1984) and ribose 5-P (Boss, G.R.: J. Biol. Chem. 259:2936-2941, 1984). The derived equilibrium relationships for each of the pentose cycle intermediates are functions of fractional powers of fructose 6-P and glyceraldehyde 3-P. These expressions indicate that large changes are required in glycolytic intermediates in order to effect relatively small changes in pentose cycle intermediates. Therefore, if equilibrium is maintained, the level of pentose cycle intermediates should not change significantly despite relatively large changes in the level of glycolytic intermediates.

Methods Employed:

Equilibrium determinations for each of the nonoxidative reactions of the pentose cycle and liver preparation were accomplished by standard laboratory techniques. Spectral assays for ribulose 5-P, xylulose 5-P, erythrose 4-P, sedoheptulose 7-P, and combined sedoheptulose 7-P and ribose 5-P were devised, as were improved enzyme assays for transketolase, transaldolase, and ribulose 5-P 3-epimerase.

Major Findings:

In ad libitum fed animals the levels of pentose cycle intermediates were described by both the tissue content of fructose 6-P and glyceraldehyde 3-P, and the equilibrium constants for the nonoxidative enzymes of the pentose cycle under a number of conditions. The same was found for starved animals. In animals meal-fed a low-fat diet these relationships were not found to be valid. An apparent disequilibrium in at least one of the major transketolase reactions resulted in large increases in the level of 6-phosphogluconate, xylulose 5-P, ribulose 5-P, and ribose 5-P.

In a related study, dehydroepiandrosterone (DHEA), an inhibitor of glucose 6-P dehydrogenase attributed with anticancer, antiobesity, and antiaging effects, was shown to have no effect on the $[NADP^+]/[NADPH]$ ratio nor did it decrease the level of any of the pentose cycle intermediates measured. It has been reported that brain DHEA levels decrease one hundredfold after acute ethanol administration (Shoemaker, W.J., Corpechot, C., Bloom, F.E., and Baulieu, E.E.: Alcoholism 8:119, 1984).

Significance to Biomedical Research and the Program of the Institute:

The changes observed in the level of pentose cycle intermediates *in vivo* represent some of the largest changes yet observed in a biological pathway. Whether these changes reflect some as yet undefined control mechanism for glucose 6-P dehydrogenase and what the effects of these changes are on other biological pathways are not clear.

Proposed Course:

The primary manifestations of Wernicke-Korsakoff's syndrome are neurological. Studies that will examine the effect of thiamine deficiency on pentose cycle intermediates in the brain are in progress.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00032-02 LM

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Identification of Glucose Metabolites in Brain and the Relationship to 2-Deoxyglucose Method

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

PI:	M.-T. Huang	Senior Staff Fellow	LM, NIAAA
Other:	R. Veech	Chief	LM, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Metabolism

SECTION

N/A

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.75	1.0	0.75

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The purity of radioactive glucose isolated as glucose-borate complex from anion exchange column chromatography was examined in this report. The eluate of the Dowex AG 1 x 8 borate column was found to be contaminated with implicity which can be removed by cation exchange chromatography. This cationic metabolite was also found to comigrate with glucose on paper chromatography. The following structural and metabolic properties were also assessed to these unidentified metabolites: that they must carry polyhydroxy group and that they are formed from glucose-6-phosphate. The time-course of the production of the anionic and cationic metabolites of glucose was compared. The amount of radioactivity in these charged metabolites seem to level off at about 7 min after the injection of labeled glucose to brain through the internal carotid artery, with the radioactivity in cationic fraction at about 1/3 of that in the anionic faction. Since metabolites of glucose in glycolysis and Krebs cycle carry negative charges, the production of the cationic metabolites may represent pathways of glucose metabolism not involved in energy production. Our results indicated that the dephosphorylation of glucose-6-phosphate may not be as large as reported previously by us. Our results also indicate that about 30% of the flux through hexokinase may be channeled through pathways not involved in energy production.

PROJECT DESCRIPTION:

Investigators:

M.-T. Huang	Senior Staff Fellow	LM, NIAAA
R. Veech	Chief	LM, NIAAA

Objectives:

We have previously studied glucose metabolism in brain in vivo by injecting [2-^{3}H]-glucose as a bolus through the internal carotid artery to brain (Huang and Veech, 1982). Since it was believed that glucose in brain is mostly consumed for energy production, labeled glucose introduced is expected to be metabolized actively through glycolysis and Krebs cycle. Radioactivity originally labeled in glucose can be expected in metabolites of these two pathways and end products such as tissue water and CO_2. Glucose as the only source of energy for brain is the basis for the 2-deoxyglucose method of brain imaging (Sokoloff et al., 1977). The latter method extrapolates regional neuronal activity to regional glucose utilization which in turn is extrapolated to hexokinase activity determined in situ with [^{14}C]-2-deoxyglucose. In our previous study (Huang and Veech, 1982), we found that the rates of metabolism for [2-^{3}H]-glucose and [U-^{14}C]-glucose are different. This data was interpreted by us as indicating active dephosphorylation of glucose-6-phosphate and futile cycling between glucose and glucose-6-phosphate in brain in vivo (Katz et al., 1975). We used tandem columns of Dowex AG 1 x 8 (formate) and Dowex AG 1 x 8 (borate) resin to isolate radioactive labeled glucose (Katz et al., 1978). Metabolites of glycolysis and Krebs cycle, mostly negatively charged, are removed first by the formate column. Glucose and other sugars are retained in the borate column as sugar-borate complexes (Khym and Till, 1952) and can thus be separated from other neutral or positively charged metabolites of glucose metabolism.

The results and conclusion of that study were challenged recently by Nelson et al. (1985) on grounds that glucose isolated from the borate column was not pure (Nelson et al., 1985). They used several chromatographic methods to purify glucose from brain extracts. With Dowex AG 1 x 8 (formate) and Dowex AG 1 x 8 (borate) columns, they found that the ^{3}H/^{14}C ratio eluted from the borate columns decreased progressively (Nelson, et al., 1985) in confirmation with our previous report. However, when the borate column eluate was subjected to cationic exchange chromatography or paper chromatography, the ^{3}H/^{14}C ratio in the eluate was raised. The time-course of the decrease in the ^{3}H/^{14}C ratio was not significantly different from that in plasma glucose. They found that up to 50% of the ^{14}C-radioactivity in the borate column eluate was removed by the cationic exchange chromatography.

In this report, various methods of purification were utilized to purify glucose from brain extract. The recovery of radioactivity and the ^{3}H/^{14}C ratio in various glucose-containing fractions were compared. The implications of the current results on our previous conclusion on the existence of an active futile cycling between glucose and glucose-6-phosphate (Huang and Veech, 1982) and on the validity of the 2-deoxyglucose method (Sokoloff et al., 1977) were discussed.

Methods Employed:

Preparation of animals for experiment was as previously described (Huang and Veech, 1982). [2-^{3}H, U-^{14}C]-Glucose (100 uCi/10 uCi) were injected through the internal carotid artery. Brains were sampled by freeze-blowing method (Veech et al., 1973).

Perchloric acid (3.6%) extract of the frozen brains were neutralized with 30% KOH. Total radioactivity in aliquot of samples before and after evaporation was determined. Radioactive metabolites of glucose were fractionated according to the charges and functional groups they carried. The isolation of the polyhydroxy metabolites of glucose on Dowex AG 1 x 8 borate column was as described (Huang and Veech, 1982). Alternatively, neutral, cationic and anionic metabolites of glucose were separated on sequential columns of Dowex AG 1 x 8 (formate) and Dowex 50 (H^{+}) resins. Aliquots of neutralized brain extracts were applied on the tandem columns. Neutral metabolites were eluted from the two columns in 10 ml of water. Cationic metabolites were eluted from the Dowex 50 (H^{+}) column with 10 ml 1 N NH_4OH. Anionic metabolites were eluted from the Dowex AG 1 x 8 formate column in 5 ml 1 N HCl.

Various glucose-containing fractions were also subjected to paper chromatography. Carrier glucose (1 umol) was co-spotted with the sample. The descending paper chromatogram was developed in a solvent system consisted of n-butanol: acetic acid: water (4 = 1 = 5). Glucose was detected by autoradiographic method or by color reaction with sodium periodate and benzidene chloride (Cifonelli and Smith, 1954). Glucose was eluted four times with 2 ml 0.1 N HCl.

Solvent in the various glucose-containing fractions was evaporated. Radioactivity was determined by external standard method.

Major Findings:

^{3}H/^{14}C ratio in various glucose-containing fraction.

Time-dependent changes in [2-^{3}H]-glucose/[U-^{14}C]-glucose ratio has often been used as an indicator for futile cycling between glucose and glucose-6-phosphate. The ratio of ^{3}H/^{14}C radioactivity in various glucose containing fractions derived from various procedures was determined. In confirmation with earlier reports (Huang and Veech, 1982; Nelson et al., 1985), the ^{3}H/^{14}C ratio in borate glucose was found to decrease progressively. The ^{3}H/^{14}C ratio in glucose-containing fractions derived from anionic and cationic exchange chromatography was found to decrease but not as much as the decrease in borate glucose. The replacement of the Dowex AG 1 x 8 borate resin with cationic exchange resin reduced the rate of decrease in ^{3}H/^{14}C ratio by two thirds. Further purification of the "neutral glucose" on paper chromatography did not affect the time-course of the decrease in ^{3}H/^{14}C ratio.

Distribution of ^{14}C-ratioactivity in neutral, acidic and cationic metabolites of glucose.

The removal of ^{14}C radioactivity by cation exchange resin was not expected, since glucose in brain is predominantly utilized for energy production and most of

the glycolytic and Krebs cycle intermediates carry negative charges. The distribution of radioactivity in neutral, anionic and cationic fractions was determined. A substantial amount of ^{14}C radioactivity was recovered in the cationic fraction. The radioactivity in the cationic and anionic fraction seems to plateau at 7 min after the injection of the isotope mixture. The radioactivity in the cationic fraction leveled off at about 30% of the radioactivity in the anionic fraction. At 10 minutes after the injection of the isotopes, the radioactivity recovered in the cationic fraction was comparable in amount to the radioactivity remained in the neutral fraction which can be accounted for as glucose.

Characteristics of the labeled cationic metabolites of glucose.

Brain extract was subjected to separation on paper chromatography. The radioactivity in spots corresponding to standard ^{14}C-glucose was eluted and determined. About 60-85% of the ^{14}C-radioactivity in the brain extract was found to co-migrate as standard glucose (Table 1).

Table 1. Percent of tissue radioactivity recovered in various glucose containing fractions.

Time after injection	Neutral glucose[1]	borate glucose[2]	PPE-glucose[3]
		(%)	
0	99.4	90.7	84.3
1	65.2	62.5	62.2
3	46.5	46.5	61.3
5	44.6	46.7	60.5
7	28.3	37.2	63.5
10	20.9	32.7	61.2

Data are average of three determinations.

[1]Derived from the 10 ml washings with water through the sequential columns of anion exchange and cation exchange resin.

[2]Derived from the eluate of the borate column.

[3]Derived from paper chromatography with 0.1 ml tissue extract.

Like borate glucose, the yield of ^{14}C-radioactivity from the paper chromatogram is greater than that of the neutral glucose fraction from ion exchange chromatography. The fraction of tissue radioactivity that can be recovered in the latter fraction was 80 to 21%. This result indicates that the cationic radioactive metabolite in the eluate of the borate column is comigrating with glucose on the paper chromatogram. The fact that these cationic radioactivity can be retained by the borate column indicates that they also possess of polyhydroxy group.

Significance to Biomedical Research and the Program of the Institute:

In a recent study to re-examine the rate of glucose-6-phosphatase in brain, Nelson et al. (1985) injected [2-^{3}H, U-^{14}C]-glucose through the internal carotid artery and sampled rat brain at different times after the injection by brain blowing, an experimental protocol essentially similar to ours as previously reported (Huang and Veech, 1982). They then purified labeled glucose by our method using sequential columns of Dowex AG 1 x 8 formate and Dowex AG 1 x 8 borate resins. Our results showing time-dependent decrease in ^{3}H/^{14}C ratio in the eluate of the borate column was duplicated by these investigators. However, when the eluate from the Dowex AG 1 x 8 borate column is chromatographed on a cation exchange column, the ^{3}H/^{14}C ratio in the effluent is increased (Nelson et al., 1985). Although these results clearly indicate the presence of cationic radioactive metabolite(s) in the eluate of the borate columns, no attempt was made by these investigators to identify these radioactive metabolite(s). Since metabolites of glycolysis and Krebs cycle are acidic, the production of the cationic metabolite(s) from radioactive glucose could represent alternate pathway for glucose metabolism in brain not involved in energy production and could therefore seriously impair the theoretical basis for the 2-deoxyglucose method (Sokoloff et al., 1977).

In this report, various methods were used to isolate radioactive glucose from brain extract. The yield of radioactivity in these glucose containing fractions were compared. It was found that the proportion of tissue radioactivity recovered in the borate glucose was significantly greater than the radioactivity recovered in the neutral glucose. This indicates that a significant portion of radioactivity in borate glucose can be removed by the cation exchange resin as reported by Nelson et al. (1985). Some structural characteristics of these radioactive metabolite(s) were indicated by these data. First, they must carry positive charge. Secondly, they must carry polyhydroxy group. Anion exchange resin in borate form has often been used to isolate radioactive glucose from tissue extract. Glucose and other polyhydroxy compounds react readily with borate ion to form several sugar-borate complexes carrying negative charges. The hydroxyl group on the 2-C of the hexose is essential for retention by the borate column. We have found that glucose, glucosamine and inositol can be retained by the borate columns while 2-deoxyglucose cannot.

The involvement of hexokinase and phosphoglucose isomerase in the production of these cationic, polyhydroxy radioactive metabolite(s) of glucose can also be inferred from our data. Since the removal of this compound raised the ^{3}H/^{14}C ratio in the glucose containing fractions, the radioactivity carried by this metabolite(s) is mainly ^{14}C radioactivity. Tritium radioactivity of [2-^{3}H]-glucose is lost through phosphoglucose isomerase. This indicating that glucose-6-phosphate is the obligatory intermediate for the production of this cationic metabolite(s) from glucose.

To sum up, the following structural and metabolic properties for this glucose metabolite(s) can be deduced from the experimental results: (1) It must carry positive charge at neutral pH environment; (2) It must carry polyhydroxy group like sugars; (3) It has very similar Rf as glucose on paper chromatography in system of butanol: Acetic acid: water; (4) It is formed from glucose-6-phosphate. Most glycolytic and dicarboxylic acid metabolites of glucose are

acidic and carry negative charges at neutral pH. Amino acids can be formed from the anionic metabolites by transamination. However, no amino acid is known to carry vicinal diol group. Hexosamines, ingredients of glycosphingolipid and glycoproteins, satisfy the structural and metabolic requirements mentioned above. The formation of hexosamines from glucose-6-phosphate is not involved in energy metabolism.

The discovery that the progressive decrease in $^{3}H/^{14}C$ ratio in the borate column eluate is not in glucose but in contaminants indicates that glucose-6-phosphate per se in brain is not as active as we have previously reported (Huang and Veech, 1982). Instead, current results suggests that glucose-6-phosphate may be converted first to hexosamine-6-phosphates and then dephosphorylated to hexosamines. As shown in Figure 2, the plateau value of the radioactivity recovered in the cationic fraction is about one-third of that in the anionic fraction. Since glucose-6-phosphate is the common precursor for the anionic and cationic metabolite, the distribution ratio of radioactivity in these two fractions could indicate relative rates of turnover of these two pathways (Shipley and Clark, 1982). The data thus indicate that the rate of metabolism from glucose-6-phosphate to pathway not involved in energy production could be as much as 30% of the rate of metabolism of glucose-6-phosphate to produce energy.

Proposed Course:

State of the art method of analysis such as high performance liquid chromatography, gas chromatography and mass spectroscopy will be employed to identify the metabolites in the eluate of the cationic columns. The significance to the 2-deoxyglucose method will be evaluated.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00033-02 LM

PERIOD COVERED
October 1, 1984 to September 30, 1985

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)*

PI:	B. Reed	Senior Staff Fellow	LM, NIAAA
Others:	M. Gerhart	Chemist	LM, NIAAA
	R. Veech	Chief	LM, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Metabolism

SECTION
Division of Intramural Clinical and Biological Research

INSTITUTE AND LOCATION
NIAAA, 12501 Washington Avenue, Rockville, MD 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.)*

Alcohol has profound effects on the hypothalamic-pituitary-gonadal axis. The effects of alcohol consumption on growth hormone secretion and action have been reviewed by De La Fuente, J.R., and Wells, L.A.: J. Clin. Psychiatry 42:270, 1981. It is known that ethanol interferes with the secretion of growth hormone (GH). Some alcoholics during withdrawal have a severely impaired GH response to insulin-induced hypoglycemia (Chalmers, R.J., Bennie, E.H., Johnson, R.H., and Kinnell, H.G.: Psychol. Med. 7:607, 1977; Eisenhofer, G., Johnson, R.H., and Lambie, D.G.: Alcoholism Clin. and Exp. Res. 8:33, 1984). Other effects include a depression of plasma GH levels following acute alcohol administration (Prinz, P.N., Roehrs, T.A., Vitaliano, P.P., Linnoila, M., and Weitzman, E.D.: J. Clin. Endocrinol. Metab. 51:759, 1980) and increased circulating GH levels in cirrhotic patients (Conn, H.O., and Daughaday, W.H.: J. Lab. Clin. Med. 76:678, 1970). At a cellular level many unanswered questions concern the mechanism of action of various growth factors. We have previously shown that in hypophysectomized animals GH is unique among the hormones tested by causing an increase in the [NADP-+]/[NADPH] ratio in liver (Veech, R.L., Nielsen, R., and Harris, R.L.: Effects of pineal and other hormones on the free [NADP-+]/[NADPH] ratio in rat liver. Frontiers of Pineal Physiology, 177-196, 1975). (This is relevant when considering the metabolic effects of ethanol, as another study has shown a transient decrease in the [NADP-+]/[NADPH] ratio in liver following ethanol administration (Veech, R.L., Guynn, R., and Veloso, D.: Biochem. J. 127:387, 1972).) Whether other growth factors may induce similar changes and the mechanism by which such changes are brought about are questions that merit investigation. To date, as a preliminary to biochemical investigations, two active fragments of GH have been prepared by recombinant DNA techniques, thus overcoming the problem of contamination of commercial GH preparations. In addition, a study has been undertaken on the early metabolic changes induced by EGF administration in vivo.

PROJECT DESCRIPTION:

Investigators:

B. Reed	Senior Staff Fellow	LM, NIAAA
M. Gerhart	Chemist	LM, NIAAA
R. Veech	Chief	LM, NIAAA

Objectives:

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

Methods Employed:

Using recombinant DNA techniques, a plasmid vector was constructed. Into this vector fragments of the human growth hormone gene were inserted. These fragments were chosen to contain the moiety responsible for binding to the receptor and other relevant active fragments. The gene fragments were expressed in E. coli by the "fusion protein" technique. The purified human growth hormone (HGH) fragments, HGH prepared by recombinant means, as well as epidermal growth factor, somatomedin, and other relevant growth factors will be tested in an in vivo system and their metabolic effects assessed.

Major Findings:

(1) A clone has been constructed containing the bacterial gene segment necessary for the production of a fusion protein.

(2) A fragment of HGH containing the determinants required for binding and purported somatomedin-like activity (Liberti, J.D.: Biochim. Biophys. Acta 675:239, 1980) has been successfully fused to the bacterial gene fragment within the plasmid vector. An abstract on the recombinant DNA methods employed entitled "Peptide expression in E. coli by gene fusion with the chloramphenicol acetyl transferase gene" has been submitted to the International Symposium of Biochemistry in Amsterdam, and a manuscript is in preparation.

(3) The resulting fusion protein has been successfully extracted and purified. Details of the purification will be presented at the Symposium of Protein Chemists in San Diego later this year.

(4) In the studies on the in vivo effects of EGF, several previously unreported acute effects of this growth factor have been discovered. The relevance of these findings in relation to the mode of action of EGF is currently being investigated.

Significance to Biomedical Research and the Program of the Institute:

Chronic alcohol consumption is known to affect the secretion of GH; whether alcohol has similar effects on the secretion or action of other growth factors is unclear. By investigating the basic mechanism by which GH or other growth factors act at a cellular level, new insight into the pathophysiology of some of the medical complications of alcoholism may be gained.

Proposed Course:

We are trying to increase the yield of the HGH peptides by various recombinant DNA techniques designed to stabilize the fusion protein in the bacterial cells. The purification of the fusion protein is being optimized. A further modification of the expression vector at the DNA level is planned to allow efficient release of the HGH moiety from the fusion protein. The cloned fragments free of other pituitary protein contamination will be tested *in vivo* for their effects on various liver metabolites and enzyme activities, and their primary intracellular action will be assessed and thus used as a model for GH action. Similar metabolic studies are also being undertaken with commercially available Epidermal Growth Factor. These studies will be extended to other growth factors and, once identified, the effect of ethanol on these key reactions will be examined.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00020-07 LM

PERIOD COVERED
October 1, 1984 to September 30, 1985

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

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

PI:	W. Schaffer	Senior Staff Fellow	LM, NIAAA
Other:	R. Veech	Chief	LM, NIAAA

COOPERATING UNITS *(if any)*
Clinical Endocrinology, NIADDK, NIH (V. Nikodem and M. Magnuson); Middleton Memorial Veterans' Hospital, Madison, WI (Y. Chen); Endocrine Section, Dept. of Medicine, U. Minnesota (C.N. Mariash)

LAB/BRANCH
Laboratory of Metabolism

SECTION
N/A

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.75	1.25	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 chronic consumption of ethanol leads to hepatic hypermetabolism (Videla, Bernstein, and Israel: Biochem. J. 134:507-514, 1974). The hypermetabolic state is thought to result in regional hypoxia and consequent central lobular necrosis in the liver of chronic alcoholics. Although careful studies have indicated that many of the data originally reported by Videla et al. could not be reproduced (Schaffer, Denckla, and Veech: Alcoholism, Clin. and Exp. Res. 5:192-197, 1981), it remains possible that thyroid action on the liver is modified after long-term ethanol ingestion by attenuating the action of T-3. Previous work by Denckla (J. Clin. Invest. 53:572-581, 1974) has indicated that extracts of bovine pituitaries have the capacity to lower the response of whole-animal minimal oxygen consumption (MOC) to thyroid hormones. By using primary cultures of rat hepatocytes, it has been possible to show that one active ingredient of the pituitary extracts is growth hormone (GH). GH lowers the response of hepatocyte malic enzyme (ME) and glycerol-3-phosphate dehydrogenase (G-3-PDH) and fatty acid synthase (FAS) activities to T-3, insulin, dexamethasone, and glucose after 6 days in culture. Since episodic secretion of GH has been reported to be blunted by ethanol (Prinz et al.: J. Clin. Endocrinol. Metab. 51:759-764, 1980), altered secretion patterns of GH in rats chronically treated with ethanol may account for the elevated activity of G-3-PDH in the absence of changes in circulating levels of thyroid hormones (Israel, Videla, MacDonald, and Bernstein: Biochem. J. 134:523-529, 1973).

PROJECT DESCRIPTION:

Investigators:

W. Schaffer	Senior Staff Fellow	LM, NIAAA
R. Veech	Chief	LM, NIAAA
V. Nikodem	Senior Staff Fellow	CE, NIADDK
M. Magnuson	Medical Fellow	CE, NIADDK
Y. Chen	Post Doctoral Fellow	Middleton Memorial Veterans Hospital
C. Mariash	Professor	Endocrine Section, Dept. Med., U. Minnesota

Objectives:

The aim of this project is to assess the contribution of thyroid hormone action to alcoholic liver damage. We also hope to evaluate the contribution of pituitary secretions to the action of thyroid hormone in liver and to identify agent(s) in extracts from pituitaries that lower tissue responsiveness to thyroid hormones. The identified thyroid hormone desensitizing agent(s) will then be studied for their effects on gene expression and cellular metabolism.

Methods Employed:

Experiments are conducted on either intact or hypophysectomized rats. Animals are treated with hormones and/or pituitary extracts. The effects of the treatment are monitored by measurement of MOC, liver enzyme activity, or the concentration of various liver metabolites. Primary cultures of rat hepatocytes are maintained in the presence of hormones for 6 days. The effects of the treatment are monitored by measuring enzyme activity and mRNA levels.

Major Findings:

(1) No evidence for hepatic or systemic hypermetabolism was observed after chronic treatment of rats with alcohol (Schaffer, Denckla, and Veech: Alcoholism Clin. Exp. Res. 5:192-197, 1981). This finding indicates that the rationale for the currently practiced treatment of alcoholic patients with the antithyroid drug propylthiouracil is incorrect (Israel, Walfish, Orrego, Blake, and Kalant: Gastroenterology 76:116-122, 1979). This conclusion of Schaffer et al. has been verified in subsequent clinical trials (Halle, Pare, Kaptein, Kanel, Redeker, and Reynolds: Gastroenterology 82:925-931, 1982). Other workers have questioned the data of Israel et al. and the use of propylthiouracil for treatment of alcoholic hepatitis (Kaplowitz: Gastroenterology 82:1468-1472, 1982).

(2) Pure preparations of GH lower the response of ME to T_3, insulin dexamethasone, and glucose in cultured rat hepatocytes.

(3) The effects of GH in cultured hepatocytes occur at concentrations known to exist in rat blood.

(4) The effects of GH on ME and fat synthesis in cultured hepatocytes requires 3 to 4 days to fully develop. Early effects are apparent within 24 hours.

(5) The effects of GH on FAS and ME act at a pretranslational level.

(6) GH does not change the molecular size of mRNA for FAS or ME, only the level.

(7) GH lowers the incorporation of glucose, water, or acetate into triglyceride and cholesterol esters as early as 24 hours after treatment with GH.

(8) GH increases phosphoinositide synthesis within 24 hours.

Significance to Biomedical Research and the Program of the Institute:

In addition to the observation of new actions for pituitary growth hormones, the known effects of alcohol consumption of GH secretion and the known lipolytic action of GH suggest that GH may have a role in the attendant pathology of chronic ethanol consumption.

Proposed Course:

We are investigating the molecular mechanism for the observed action of GH on cultured liver tissue.

Publications:

Schaffer, W.T., Mehlman, M.A., and Veech, R.L.: Effects of 5-5' diphenylhydantoin and thyroxine on phosphorylation potential and redox state of freeze-clamped rat liver. Toxicol. Indust. Health (in press).

Schaffer, W.T.: Effects of growth hormone on lipogenic enzyme activities in cultured rat hepatocytes. Am. J. Physiol. 248:E719-E725, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00023-07 LM

PERIOD COVERED

October 1, 1984 to September 30, 1985

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)*

PI:	R. Veech	Chief	LM, NIAAA
Other:	W. Gitomer	Senior Staff Fellow	LM, NIAAA

COOPERATING UNITS *(if any)*

Lab of Kidney and Electrolyte Metab., NHLBI (W. Balaban); Skeletal Biophysics Section, NDI (D. Eanes); Clinical Neuropharmacology Branch, NIMH (J. Costa)

LAB/BRANCH

Laboratory of Metabolism

SECTION

N/A

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 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 μM, 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.

PROJECT DESCRIPTION:

Investigators:

R. Veech	Chief	LM, NIAAA
W. Gitomer	Senior Staff Fellow	LM, NIAAA
W. Balaban	Research Scientist	LKEM, NHLBI
J. Costa	Medical Officer	CNB, NIMH
D. Eanes	Research Scientist	SBS, NDI

Objectives:

Most emphasis in the research concerning alcohol metabolism has centered on the possible reactions and potential significance of acetaldehyde. While of some theoretical interest, the thermodynamic characteristics of the reactions involved force the acetaldehyde concentration to remain very low, of the order of 2 μM. In contrast, blood levels of 2 mM acetate are routine after drinking even very small quantities of ethanol since acetate is the primary product of ethanol metabolism. So far there has been very little systematic study of the metabolic effects of acetate. This study was therefore begun to elucidate the consequences of acetate metabolism on the major metabolic pathways, particularly its effect on the coenzymes and the PPi-related cofactor couples.

Methods Employed:

The study of the various pathways involved has required a whole series of new analytical methods for use in animal tissue. These methods are:

(1) an enzymatic method for the spectrophotometric measurement of ribulose 5-P and ribose 5-P.

(2) an HPLC method for measurement of purine and pyrimidine nucleotides.

(3) an HPLC method for measurement of purine and pyrimidine bases.

(4) an enzymatic method for measurement of phosphoribosylpyrophosphate.

(5) an HPLC method for measurement of all the coenzyme A-derived nucleotides.

(6) a carbon furnace atomic absorption method for measurement of Mg and Ca.

(7) a surface coil method for obtaining NMR signal from PPi in rat liver in vivo in collaboration with the National Heart Institute.

(8) a survey of histochemical localization of PPi and Ca using electron microscopy.

(9) a method for measurement of mitochondrial contents of PPi and Ca in isolated rat hepatocytes.

(10) an enzymatic method for measurement of inorganic PPi.

(11) a radioautographic method for semiquantitative determination of the effects of acetate on cerebral energy metabolism.

(12) digitonin fractionation of hepatocytes.

Eight of these twelve methods were developed in this laboratory.

Major Findings:

The administration of acetate to starved rats causes (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. The Ca and PPi accumulate in the mitochondria as an insoluble salt.

In the area of methods development, this project has high significance. Having previously developed the first practical method to measure inorganic PPi in tissue or body fluids, we have now developed: (1) HPLC methods for measurement of most of the tri, di, and monophosphates, (2) an HPLC method for measurement of purine and pyrimidine bases, and (3) an HPLC method for measurement of the majority of tissue coenzyme A derivatives. These methods already are being used in a number of other research areas.

Significance to Biomedical Research and the Program of the Institute:

The elevation of PPi to 2 to 4 mM levels by acetate raises fundamental questions in a number of areas of metabolism. The elevation of liver and hence blood glucose by the cytoplasmic generation of PPi suggests the importance of this cofactor not only as a regulator of glucose, but as a previously unconsidered factor in diabetes.

Perhaps even more fundamental is the effect that PPi is known to have on DNA and RNA metabolism. Inorganic PPi at 2 mM levels is known to reverse DNA polymerase, with potentially marked effects on the genetics of the cells involved. In that regard, butyrate has long been known to detransform malignant cells, that is, to make these cells appear to behave as normal nonmalignant tissue. While the roles of acetylation of nuclear histones has heretofore been accepted as a partial explanation of this phenomenon, the role of PPi in detransformation will now have to be considered.

Elevation of intracellular PPi exerts the largest and most rapid changes in cellular calcium contents yet observed. Intracellular Ca^{2+}, along with cyclic-AMP, is the major cellular regulator of a multiplicity of reactions, from hormone actions of all sorts to malignant transformation by oncovirus. The role of PPi in controlling cellular Ca^{2+} will now have to be considered.

Finally, a number of disease states found in alcoholics have never been looked at from the point of view of the effects of acetate per se. Particularly obvious is the association of gout with alcohol intake in those patients with a genetic susceptibility to hyperuricemia.

Proposed Course:

Six papers are in preparation about the work described above. The relationship of PPi to a number of fundamental metabolic syntheses including glucose, protein, nucleic acids, and urea will be examined from a basic point of view and from the point of view of abnormalities induced by the presence of acetate in the alcoholic.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 00028-03 LM

PERIOD COVERED
October 1, 1984 to September 1, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of the Salvage Reactions in the Regulation of Purine Metabolism

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

PI:	R. Veech	Chief	LM, NIAAA
Other:	T. King	Research Chemist	LM, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Metabolism

SECTION
N/A

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.1	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.)*

This project compared thermodynamic data obtained in vitro for the purine salvage reactions to the measured steady-state levels of purine and pyrimidine nucleobases, nucleosides, nucleotides, and related cofactor compounds in vivo. (The hypothesis is that the purine salvage enzymes catalyze reactions that approach near-equilibrium, in vivo, and thus that rates of synthesis of the necessary cofactors such as ribose 1-phosphate (R1-P), phosphoribosylpyrophosphate (PRPP), inorganic phosphate (Pi), and inorganic pyrophosphate (PPi), could profoundly influence the relative amounts of free bases and nucleosides available for nucleotide and urate production.)

Current methods for the extraction and quantitation of purine and pyrimidine nucleobases and nucleosides as well as for R1-P and PRPP are suspect and have led to confusion and possibly misinterpretation of scientific data. Therefore, we began by attempting to improve on current methods for measuring the aforementioned metabolites in liver extracts. A superior high-performance liquid chromatographic procedure was developed for measuring purine and pyrimidine nucleobases and nucleosides in rat hepatocytes. Progress was also made on improving techniques for the extraction and quantitation of R1-P and PRPP.

The apparent equilibrium constants for the purine salvage reactions catalyzed by purine nucleoside phosphorylase were completed as well as for the ancillary reactions, phosphoribomutase and 5' nucleotidase. The equilibrium constant for the phosphoribosyltransferases will be completed once PRPP can be measured. Once these in vivo measurements are made it can be determined whether the salvage reactions approach near-equilibrium in vivo and, if so, whether it will be possible to assess the influence of fluctuations in the magnitude of the cofactor couples PRPP/PPi and R1-P/Pi on the rates of nucleotide, nucleic acid, and uric acid synthesis in a variety of cell types. This project has been terminated.

Publications:

Reiss, P.D., Zuurendonk, P.F., and Veech, R.L.: Measurement of tissue purine, pyrimidine, and other nucleotides by radial compression high-performance liquid chromatography. Anal. Biochem. 140:162-171, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00024-07 LM

PERIOD COVERED
October 1, 1984 to September 30, 1985

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)*

PI:	R. Veech	Chief	LM, NIAAA
Other:	J. Casazza	Senior Staff Fellow	LM, NIAAA

COOPERATING UNITS *(if any)*
Bureau of Epidemiology, CDC, Atlanta, GA (S. Thacker); Tott's Gap, Med. Res. Found. (S. Wolf); Dept. of Preventive Med., Harvard Medical School, Boston, MA; Dept. Biological Chem., Univ. Mich., Ann Arbor, MI (D. Koop); Dept. Int. Med., Keio Univ., Japan (Ishii)

LAB/BRANCH
Laboratory of Metabolism

SECTION
N/A

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)

☒ (a) 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 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-l, or meso 2,3-butanediol to the 5 μM range.

In the rat, two pathways of butanediol formation have been demonstrated. The first (Veech et al.: Curr. Top. Cell Regul. 18:151-179, 1981) 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 genetic differences in the induced 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.

PROJECT DESCRIPTION:

Investigators:

R. Veech	Chief	LM, NIAAA
J. Casazza	Senior Staff Fellow	LM, NIAAA
S. Thacker	Chief	Bureau of Epidemiology, CDC
S. Wolf	Director	Tott's Gap, Med. Research Foundation
D. Rutstein	Professor	DPM, Harvard Med. School
D. Koop	Asst. Professor	Dept. Biological Chemistry, U. Michigan, Ann Arbor
H. Ishii	Professor	Dept. Int. Med., Keio Univ.

Objectives:

The purpose of this series of studies was to determine if alcoholics metabolize alcohol by a different pathway with different pathway controls or with different metabolic consequences than do nonalcoholics. 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, R.L.: N. Engl. J. Med. 298:1140-1141, 1978). 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. Synthesis of chemical intermediates of the diol pathway was accomplished by standard techniques of organic chemistry.

Major Findings:

Clinical methods. A new method for measuring the formation of 1-hydroxyacetone (acetol) has been developed (Casazza, J.P., and Fu, J.L.: Anal. Biochem. 148: 344-348, 1985), which allows measurement of this compound down to concentrations

of 2-5 μM in serum. This method is based on derivatization of acetol with dinitrophenylhydrazine and extraction of the resulting hydrazone into a nonpolar phase. Acetol has been identified in human serum during a prolonged fast, in acetone-treated rats and rabbits, and in rats during severe ketoacidosis.

Clinical studies. In a blind study, seven Japanese medical students were given 0.5 g ethanol/kg body weight and blood samples were drawn over a 2-hour period. Of these seven, four were "flushers," a genetic isolate thought to have either nonexistent or nonfunctional low Km aldehyde dehydrogenase. This absence of a low Km aldehyde dehydrogenase suggests that "flushers" would have much higher levels of blood acetaldehyde than any other known group of humans. The concentration of 2,3-butanediol was 5 μM in all samples (Casazza, J.P., Ishii, H., and Veech, R.L.: Alcohol 2:44-47, 1985). This study lends no support to the hypothesis that elevated blood acetaldehyde results in the production of 2,3-butanediol.

Metabolic studies. During the past year, the production of 1,2-propanediol has been shown in rats given a Lieber-DeCarli ethanol diet; no 1,2-propanediol was found in animals pair-fed the control diet (Casazza, J.P., and Veech, R.L.: Biochem. Biophys. Res. Commun. 129:426-430, 1985). The production of 1,2-propanediol was accompanied by a fourteenfold increase in 3-hydroxybutyrate and an eightfold increase in both microsomal acetone and acetol monooxygenase activity. Serum 1,2-propanediol decreased when the level of blood ketones decreased. These data are consistent with the mechanism proposed by Casazza et al. for 1,2-propanediol production in acetone-treated animals (Casazza, J.P., Felver, M.E., and Veech, R.L.: J. Biol. Chem. 259:231-236, 1984). No 2,3-butanediol was observed in any of the ethanol-treated animals.

We have also shown in rabbit the identity of P-450 LM 3a, the major ethanol inducible P-450, and the microsomal acetone and acetol monooxygenase. In rabbit, chronic acetone feeding results in the largest induction of this enzyme yet found. Chronic acetone feeding in rabbit also results in the production of acetol and 1,2-propanediol. These data suggest that the production of 1,2-propanediol in alcoholics may be due to a similar P-450 enzyme. It is not clear that this is the case. Although alcoholic ketoacidosis is known, its incidence is much lower than the measured occurrence of 1,2-propanediol (Rutstein, D.D., Veech, R.L., Nickerson, R.J., Felver, M.E., Vernon, M.E., Needham, L.L., Kishore, P., and Thacker, S.B.: Lancet ii:534-536, 1983).

Further studies aimed at defining the mechanism of production of 2,3-butanediol in acetone-treated animals are in progress. Several proposed intermediates have been synthesized isotopically, and means have been devised for the isolation of these compounds from biological material and for their measurement.

Significance to Biomedical Research and the Program of the Institute:

These series of studies identify for the first time new compounds that either are metabolites of alcohol itself or are induced by its ingestion. The data to date are still compatible with, but not conclusive of, the possibility that the enzymes responsible for the elevation of 2,3-butanediol represent an inherited metabolic abnormality distinguishing alcoholics from nonalcoholics.

Proposed Course:

Work will continue on elucidating the metabolic pathways of diol metabolism in humans. No suitable metabolic studies have so far been possible in humans for administrative reasons. Until such studies are completed, the full importance of these findings will not be known in regard to the etiological complications. As an objective "magic bullet" indicative of alcoholism, however, this family of compounds could constitute a valuable diagnostic tool. The CDC, the Harvard School of Public Health, and the National Academy of Sciences Twin Registry are now engaged in a collaborative project to determine in identical twins, one of whom is alcoholic, the extent to which diol production is the result of predetermined genetic factors and to what extent it is induced. At the same time the Tott's Gap group, in collaboration with an alcohol treatment center, is investigating the relationship between the presence of diols in the blood of alcoholics and cerebral functioning.

Publications:

Casazza, J.P., Ishii, H., and Veech, R.L.: The measurement of 2,3-butanediol and 1,2-propanediol in "flushing" and "non-flushing" Japanese. Alcohol. 2:44-47, 1985.

Casazza, J.P., and Veech, R.L.: The measurement of acetol in serum. Biochem. Biophys. Res. Commun. 129:426-430, 1985.

Casazza, J.P., and Fu, J.L.: The measurement of acetol in serum. Anal. Biochem. 148:344-348, 1985.

Annual Report of the Laboratory of Preclinical Studies
National Institute on Alcohol Abuse and Alcoholism
October 1, 1984 to September 30, 1985
Forrest F. Weight, M.D., Chief

Introduction

In fiscal year 1985, the Laboratory of Preclinical Studies has continued to make significant progress in its investigations. The Laboratory's research has been directed primarily toward establishment of firm biological foundations upon which the actions of ethanol and their clinical relevance can be interpreted. One of the goals of the Laboratory is to elucidate the pathophysiologic mechanisms involved in alcohol intoxication, dependence, and withdrawal in order to provide information relevant to the causes, processes, and treatment of alcohol abuse and alcoholism. The studies in the Laboratory are primarily in two broad areas: (1) studies on the systemic effects of alcohol intoxication, dependence, and withdrawal; and (2) studies on the cellular and molecular basis of alcohol's actions. These two areas provide the organizational basis for this report.

1. Studies on the Systemic Effects of Alcohol Intoxication, Dependence, and Withdrawal

It is well known that alcohol abuse in humans is correlated with alterations in the function of several organ systems including the immune system, the central nervous system, and the constituents of blood. We have investigated the effects of ethanol intoxication and dependence on these systems in order to determine the alterations in function that are due to ethanol, as well as to determine the mechanisms by which such effects occur.

A. Immune System

Alcoholics have a higher incidence of various infections than nonalcoholics, raising the possibility that prolonged and excessive consumption of alcohol may lower some aspect of the body's defense mechanisms. We have investigated the effects of ethanol intoxication and dependence on the immune system of the rat in collaborative experiments with Drs. T. Jerrells and R. Meagher (Walter Reed Army Institute of Research), and Dr. M. Eckardt (Laboratory of Clinical Studies, NIAAA). We have found that lymphocytes in spleen, thymus, and peripheral blood decrease markedly in number after treating the animals with ethanol. In addition, the remaining lymphocytes in spleen and peripheral blood show an impaired ability to divide in response to mitogenic stimuli. Immunization studies with T cell-dependent and T cell-independent antigens indicate that the effect of ethanol appears to be at the T-cell level. Because lymphocytes are involved in the body's defense against infectious diseases, the effects of ethanol on lymphocyte number and function may contribute to the increased incidence of infections in alcoholics. A paper entitled "Ethanol induced alterations in the immune system: Loss of lymphocyte numbers and functions after ethanol administration in rats" has been submitted for publication.

B. Central Nervous System

One important aspect of understanding the effects of ethanol intoxication, dependence, and withdrawal on brain function is determining the localization of the effects on various brain regions. The development of the 2-deoxyglucose technique has provided a method for studying localized changes in brain metabolism. We have used this technique to study the effects of acute and chronic ethanol administration and withdrawal on glucose metabolism in rat brain. We found that in physically dependent animals undergoing the withdrawal syndrome, increases in glucose utilization are observed particularly in sensory and motor brain regions. A paper reporting these observations is in press in Brain Research (Eckardt, MJ, Campbell, GA, Majchrowicz, E, Wixon, HN and Weight, FF: Cerebral 2-deoxyglucose uptake in rats during ethanol withdrawal and postwithdrawal.) Comparision of these observations with data from animals undergoing phenobarbitol and diazepam (Valium) withdrawal reveals that although there is a generalized increase in 2-deoxyglucose uptake during withdrawal from each of these depressants, there are both similarities and differences in the localized areas of uptake. In addition, we found that although in control animals the acute administration of diazepam did not significantly alter glucose uptake, in animals withdrawing from ethanol the administration of diazepam prevented the increased glucose uptake usually observed during withdrawal. This observation correlates with the absence of the behavorial signs of withdrawal in diazepam-treated animals. These observations provided an essential foundation for future investigations on the pathophysiologic alterations of brain function associated with the withdrawal syndrome. Moreover, they suggest the possible use of the 2-deoxyglucose technique for screening new drugs that might be useful in the treatment of ethanol withdrawal.

C. Blood Chemistries

Clinically, abnormalities in the function of many of the body's tissues and organs are reflected in alterations in the value of various blood chemistries. To obtain an index of the effects of ethanol on different tissues, we have studied the effects of ethanol intoxication and dependence on blood chemistries in the rat. Analysis of the blood chemistry profiles has revealed a significant elevation in blood cholesterol in dependent-intoxicated animals. The profile of blood plasma lipoproteins indicates that it is the high density lipoproteins that are elevated in the dependent-intoxicated animals. Since an elevation of high density lipoproteins has been reported to be associated with ethanol consumption in humans, these observations may provide an experimental model for investigating the relationship between ethanol and serum lipoproteins.

2. Studies on the Cellular and Molecular Basis of Ethanol's Action

A. Nerve Cell Degeneration in Brain

Brain damage occurs in over 60 percent of alcoholics. Recent studies have suggested that the cerebral atrophy and deterioration of brain function that results from chronic alcohol ingestion may be due to a loss of neurons in

the brain. We have studied the degeneration of neurons associated with ethanol administration in collaboration with Dr. R. Switzer (Univ. Tenn.). Using a silver stain to detect degenerating neurons in rat brain, we have found evidence for degenerating neurons after treating animals with intoxicating doses of ethanol for 4 days. The degenerating neurons were located primarily in the lateral and perirhinal cortex, as well as in the ventral hippocampus. In some cases, degenerating neurons were also observed in the lateral amygdala. The demonstration of neuronal degeneration induced experimentally by the administration of ethanol provides an experimental paradigm for investigating the cellular and molecular mechanisms involved in the ethanol-induced degeneration of neurons.

In an attempt to understand more about the mechanisms involved in ethanol-induced brain damage, we studied the effect of thiamine deficiency on rat brain. It is known that one type of alcohol-induced brain damage, Wernicke-Korsakoff's encephalopathy, results from thiamine deficiency associated with alcoholism. We found that the thiamine-deficient animals exhibited a breakdown of the blood-brain barrier as indicated by small hemorrhages and an accumulation of blood proteins such as albumin, fibrinogen, and hemoglobin. This suggests that the breakdown of the blood-brain barrier may contribute to the brain damage associated with thiamine deficiency.

B. Nerve Cell Excitability

Ethanol is classified pharmacologically as a central nervous system depressant. The cellular mechanisms involved in ethanol's effects on nervous system excitability, however, are poorly understood. Recent studies have suggested that ethanol may affect nerve cell excitability by altering membrane ion fluxes. However, at present, direct evidence that ethanol affects membrane ion channel function is lacking. We have studied neuronal membrane ion currents using two recently developed biophysical methods, patch-clamp recording and single-electrode voltage clamp recording. We have characterized both sodium and calcium currents in mammalian neurons, and are currently studying the action of ethanol on those currents. Further analysis of the effects of ethanol on membrane ion currents should increase our understanding of the ionic basis of ethanol's effects on nerve cell excitability. A paper characterizing potassium currents in hippocampal CA3 neurons was recently published (Zbicz, KL and Weight, FF: Transient voltage and calcium dependent outward currents in hippocampal CA3 pyramidal neurons. J. Neurophysiol. 53:1038-1058, 1985). In addition, papers with the following titles have been submitted for publication: "Two kinetically distinguishable components of the transient outward current in sympathetic neurones of Rana catesbeiana"; and, "Na+ and CA2+ currents of acutely isolated adult rat nodose ganglion cells".

C. Synaptic and Neurosecretory Mechanisms

Previous studies indicate that ethanol can alter both neurotransmitter release and neurosecretion. The mechanisms involved in these effects, however, have not been elucidated. In earlier studies in this Laboratory, we found that ethanol alters calcium metabolism in synaptosomes (pinched off nerve terminals), suggesting that the effects of ethanol on neurotransmitter release may result from alterations in cellular calcium metabolism. Since

it is not possible, at the present time, to study calcium channels or intracellular calcium signals in nerve terminals, we are studying these phenomena in two neurosecretory cell lines. In the mouse pituitary cell line, AtT-20, we are using patch clamp recording to study the regulation of the calcium channel in relation to ACTH release, and in the rat chromaffin cell line, PC12, we are investigating the interrelationships of intracellular calcium mobilization, phosphoinositide metabolism, and norepinephrine release. The effect of ethanol on these neurosecretory mechanisms is currently being studied. These investigations hold the promise of increasing our knowledge of the mechanisms involved ethanol's effects on neurotransmitter release and neurosecretion. Several papers on synaptic mechanisms have been published (Weight, FF, Postsynaptic mechanism in long-lasting potentiation of synaptic transmission, In: Neurobiology of Learning and Memory, Eds: JL McGaugh, G Lynch, and NM Weinberger, Guilford Publ., New York, 1984, pp. 491-499; Campbell, GA, Eckardt, MJ and Weight, FF, Dopaminergic mechanisms in subthalmic nucleus of rat: analysis using horseradish perioxidase and microiontophoresis, Brain Research 333:261-270, 1985; Doller, HJ and Weight, FF, perforant pathway evoked long-term potentiation of CA1 neurons in the hippocampal slice preparation, Brain Research 333:305-310, 1985; and, Schofield, GG and Weight, FF, Single acetylcholine channel currents in sympathetic neurons, Brain Research 343:200-203, 1985). In addition, papers with the following titles have been submitted for publication: "Antagonists discriminate muscarinic excitation and inhibition in sympathetic ganglion"; "MPTP causes a non-reversible depression of synaptic transmission in mouse neostriatal brain slice"; and, "The pathway for the slow inhibitory postsynaptic potential in bullfrog sympathetic ganglia."

D. Cellular Calcium Metabolism

The fetal alcohol syndrome indicates that alcohol can affect developing tissues. However, the cellular mechanisms of these effects are not known. To determine the effects of ethanol on the early stages of cell division, we used light microscopy to study the development of fertilized sand dollar eggs. We found that ethanol alters the cytoplasmic organization of the cells and inhibits cell division. Similar results were obtained by injecting micromolar concentrations of calcium ions, suggesting that the effects of ethanol on cell division may result from an elevation of cytosolic calcium concentration. These studies suggest that the preparation may provide an experimental model for elucidating the mechanisms involved in the effects of ethanol on the early stages of development. Several papers relating to cellular calcium metabolism have been published or are in press (Pande, U and Pant, HC, Effect of ethanol on calcium metabolism in rat erythrocyte ghosts, Physiol. Chem. Phys. and Med. NMR 16:463-467, 1984; Pande, U and Pant, HC, Evaluation of calcium-arsenazo III stoichiometry in the presence and absence of EGTA at various pH, Analytical Letters (in press); Pant, HC, Majchrowicz, E, and Virmani, M, Cerebral alteration in calmodulin levels associated with the induction of physical dependence upon ethanol in rats, Brain Research (in press); and, Virmani, M, Majchrowicz, E, Swenberg, CE, Gangola, P and Pant, HC, Alteration in calcium-binding activity in synaptosomal membranes from rat brain in association with physical dependence upon ethanol, Brain Research (in press)).

E. Protein Phosphorylation

The phosphorylation of proteins is thought to be one of the important molecular mechanisms involved in cellular regulation. Certain neurofilament proteins are known to be highly phosphorylated, but little is known about the endogenous kinase activity that phophorylates these proteins. We have characterized the protein kinases that phosphorylate the cytoskeletal proteins in several tissues and found that the kinase activity is increased by ethanol. This observation provides an experimental basis for investigating the effects of ethanol on the structure and function of these enzymes and cytoskeletal proteins. Three papers have been published (Gallant, PE, Pant, HC and Gainer, H, Distribution of acid protease activity in squid nervous system, J. Neurochemistry 42:590-593, 1984; Gainer, H, Gallant, PE, Gould, R and Pant, HC, Biochemistry and metabolism of the squid axon, Edited by P. Baker, Current Topics in Membranes and Transport 22:57-90, 1984; and, Pant, HC and Virmani, M, Calcium regulation of magnesium dependent phosphorylation of human erythrocyte ghost spectrin, Physiol. Chem. Phys. Med. NMR 84:283-292, 1984). In addition, a paper with the following title has been submitted for publication: "Phosphorylation and calcium activated proteolysis of neurofilament proteins in the squid giant axon."

Conclusions

The preceding summary indicates the progress that the Laboratory of Preclinical Studies has made in elucidating the cellular and molecular basis of ethanol's actions. The progress made by the Laboratory gives every reason for optimism that in the future we will be able to greatly further our knowledge of the acute and chronic actions of ethanol, and may start the search for documentable cellular pathophysiology of alcohol abuse.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00475-02 LPS

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Blood Chemistry Profiles and Ethanol Dependence

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

PI:	E. Majchrowicz	Research Chemist	LPS, NIAAA
Others:	E. Lamoreaux	Computer Programmer	LPS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Preclinical Studies

SECTION
N/A

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

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.)*

The blood plasma lipoprotein profile in ethanol-treated rats was studied by determining the levels of very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) after a single dose (6 g/kg) of ethanol in ethanol-dependent rats. Three characteristic neurobehavioral groups of rats were distinguished during the ethanol withdrawal period: (1) the dependent intoxicated (prodromal) group, (2) the overt withdrawal syndrome group, and (3) the convulsive seizure group. No significant changes in any lipoproteins were found up to 24 hours after the single dose of ethanol. In the ethanol-dependent rats, VLDL decreased, HDL increased, and no significant change in the LDL levels occurred. These data suggest that elevation of HDL in the blood of rats treated with ethanol for 4 days is similar to that found in human alcoholics.

This project has been completed. A manuscript is being prepared for publication.

PROJECT DESCRIPTION:

Investigators:

E. Majchrowicz	Research Chemist	LPS, NIAAA
E. Lamoreaux	Computer Programmer	LPS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA

Objectives:

Consumption of alcoholic beverages results in impaired lipid metabolism in the liver, hyperlipemia, and derangement in the composition and distribution of blood plasma lipoproteins. Clinical and epidemiological studies have demonstrated that ethanol consumption is inversely related to the occurrence of coronary heart disease. Atherosclerosis is characterized by the formation of atherosclerotic plaques in the intima layer of the artery wall. Growth of plaques in the coronary artery may result in partial or complete obstruction of the blood flow to the heart muscle, possibly followed by anginal pain (coronary heart disease) or sudden death. Atherosclerotic plaques are filled with scavenger cells that ingest large amounts of cholesterol and cholesterol esters and subsequently become "foam cells." The formation of atheroma depends on the abundance of blood cholesterol, which is distributed throughout the body in the LDL and other lipoproteins. The evidence is that HDL are cholesterol scavengers that retard the atherosclerotic plaque formation by removing cholesterol from atheromatous tissue and transporting it to the liver. In the liver, the cholesterol-carrying HDL are taken up by receptor-mediated endocytosis, during which the cholesterol is converted into bile acids and excreted into the gut. It has been postulated that HDL competes with LDL for the specific receptor controlling the endocytosis. Thus increased blood levels of HDL induced by the ingestion of alcoholic beverages may be an underlying mechanism in the protective action of ethanol against atherosclerosis. This study was undertaken using our animal model of physical dependence on ethanol in order to gain insight into the metabolic process just described.

Methods Employed:

The basic methodology involved the induction of physical dependence upon ethanol in rats using a previously established technique (Majchrowicz, E.: Psychopharmacologia 43:245, 1975). Physical dependence was induced by intragastric administration of 20% ethanol in Sustacal at 8 to 11 g/kg/day in 6 to 10 fractional doses for 4 days. After decapitation, blood was collected from ethanol-dependent rats at various intervals of the ethanol withdrawal period and after a single oral dose (6 g/kg) of ethanol. Lipoproteins were estimated by agarose gel electrophoresis conducted at 110 volts for 35 minutes in a 0.05 M barbital buffer at pH 8.6. Following electrophoresis, the lipid moiety of the lipoproteins was stained with Fat Red 7B stain and scanned on a Beckman densitometer with an integrator. The percentage of the dye taken up by each of the three lipoprotein bands was determined. The principal lipoproteins determined by agarose gel electrophoresis are approximately equivalent to those separated on the basis of density ultracentrifugation: high-density lipoprotein or HDL, low-density lipoprotein or LDL, and very low-density lipoprotein or VLDL.

Major Findings:

Lipoproteins were estimated during the following time intervals of the ethanol withdrawal period: (1) the dependent intoxicated (prodromal) phase when rats were still intoxicated; (2) at 5, 10, and 15 hours after the onset of the overt signs and responses of the ethanol withdrawal syndrome; (3) while the rats were undergoing spontaneous convulsions (used immediately after the remission of the seizure episode); and (4) at 4, 8, 16, and 24 hours following the administration of 6 g/kg of ethanol. With each experimental group there was a group of control rats against which the comparisons were made.

The VLDL in the dependent intoxicated (prodromal) rats were 40% lower than those in the control rats. As the withdrawal syndrome evolved, the prodromal rat VLDL levels gradually increased. On the basis of the pattern of increase, it seems likely that in about 24 to 48 hours after the initiation of the overt withdrawal syndrome the levels of VLDL in the dependent rats would return to those in the control group. The blood levels of the LDL decreased significantly through the three stages of the withdrawal syndrome investigated. The HDL levels in the dependent rats were approximately 19% higher than those in the control rats and the HDL concentrations did not change significantly during any stages of the withdrawal period investigated. After single doses of ethanol, only small and insignificant changes in blood levels of all three major lipoproteins occurred. The response pattern of the three LP's in the blood through the various stages of the withdrawal period suggests the characteristic LDL turnover and/or utilization. The VLDL appeared to be leveling off toward the control levels within a short period of time after the initiation of the overt withdrawal syndrome. This leveling off corresponds to the time period for ethanol clearance from the blood. Both the LDL and HDL levels appeared to be readjusting to control levels at a slower rate than the VLDL. This observation is analogous to that in human subjects where it has been observed that VLDL have a rather short life span lasting only several hours, whereas the lives of the LDL and HDL are measured in several days.

Significance to Biomedical Research and the Program of the Institute:

The tolerance and physical dependence on ethanol following chronic consumption involves some as yet unidentified changes in the function of the central and peripheral nervous systems and perhaps in other organs of the body. After short-term and prolonged consumption of alcoholic beverages the blood contains, in addition to ethanol, a large variety of substances derived from ethanol or that appear as a consequence of ethanol's interaction with body tissues and organs. Numerous metabolic byproducts and tissue components are released into the blood and can be used as a measure of the derangement of the homeostatic state of the organism or of frank tissue injury. It is important to know what molecular mechanisms underlie the protective action of ethanol against coronary heart disease. Since ethanol has a relatively simple chemical structure it might be possible to use it as a model in elucidating complicated clinical conditions relating to the heart or other complications in lipid metabolism.

Proposed Course:

This project has been completed and a manuscript is being prepared for publication.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00477-02 LPS

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Ethanol and Its Metabolites During Intoxication and Physical Dependence

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
PI: E. Majchrowicz Research Chemist LPS, NIAAA

COOPERATING UNITS *(if any)*
Biological Psychiatry Branch, NIMH (E. Tamborska)

LAB/BRANCH
Laboratory of Preclinical Studies

SECTION
N/A

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

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.)*

The blood acetate clearance curve had a biphasic character during the ethanol withdrawal period in ethanol-dependent rats. Blood acetate concentrations remained relatively stable for 15 to 20 hours, during which time blood ethanol was removed in a rectilinear fashion. Blood acetate concentrations remained on a plateau at approximately 8 to 9 mg/dL. When blood ethanol concentration decreased to approximately 80 to 50 mg/dL, blood acetate began to decrease very rapidly. The leveling off of the blood acetate concentrations at a plateau suggests that blood acetate concentrations are probably related to the rate of ethanol oxidation. The blood acetate curve of naive rats after a single dose of ethanol had a shape similar to the acetate curve in rats rendered physically dependent on ethanol. Concentrations of acetate similar to those in ethanol-dependent rats have been previously found in human alcoholics participating in a free drinking paradigm. This project has been completed. A manuscript for publication is being written.

PROJECT DESCRIPTION:

Investigators:

E. Majchrowicz	Research Chemist	LPS, NIAAA
E. Tamborska	Visiting Fellow	BPB, NIMH

Objectives:

Acetate plays a central role in the general metabolism of plants and animals. Numerous metabolic processes involving the interaction of carbohydrates, fats, and proteins are either directly or indirectly interconnected through an active derivative of acetate (i.e., acetyl-CoA) with the citric acid cycle, glycolysis, and other biosynthetic processes. In ruminants, acetate and other short-chain aliphatic fatty acids are derived from the microbial fermentation of the ingested carbohydrates, whereas in primates, acetate is derived exclusively from endogenous sources. A typical example of an increased supply of acetate produced in the body from an exogenously administered substrate is the administration of ethanol or other alcoholic beverages. Oxidation of ethanol in the liver results in the formation of acetaldehyde, which is instantly oxidized to acetate. Each of the three pathways of ethanol oxidation (alcohol dehydrogenase, catalase, or microsomal ethanol oxidizing system [MEOS]) eventually results in the formation of acetate.

After administration of alcoholic beverages, the formation of acetate in the liver exceeds the liver's capacity to metabolically utilize the acetate. Thus, most of the acetate formed in the liver is released into the systemic blood and subsequently is distributed throughout the body and used in a variety of metabolic processes. For the most part, acetate is oxidized to carbon dioxide, water, and energy. The ethanol-derived acetate merges with the exogenous acetate pool and subsequently most acetate metabolizing pathways become saturated, diverting the available coenzymes and enzymes away from the metabolism of endogenous substrates.

Our earlier studies (Majchrowicz, E., and Quastel, J.H.: Can. J. Biochem Physiol. 39:1895, 1961), which showed that ethanol and other short-chain aliphatic alcohols severely inhibit the metabolism of acetate in liver slices, have a number of implications for the formation and disposition of ethanol-derived acetate. In addition, our studies in human subjects and experimental animals showed that there is an elevation of blood acetate concentration after administration of ethanol and that in rats ethanol inhibits exogenously administered acetate from the blood. In order to gain insight into the possible fate of ethanol-derived acetate, we studied blood acetate and blood ethanol levels in rats rendered physically dependent on ethanol.

Methods Employed:

Physical dependence on ethanol was induced in male Sprague-Dawley rats by oral administration of 20% ethanol in Sustacal for 4 days as previously described (Majchrowicz, E.: Psychopharmacologia 43:245, 1975). On the day following the last dose of ethanol, the rats were observed at 1-hour intervals starting at 7 a.m. Blood samples were taken from the tail vein at 1-hour intervals at various stages of intoxication for gas chromatographic determination of ethanol and

acetate as described in Majchrowicz, E., and Hunt, W.A.: Biochem. Pharmacol. 27:128, 1978.

Major Findings:

The blood acetate clearance curve had a biphasic character in ethanol-dependent rats during the ethanol withdrawal period. After ethanol withdrawal, blood acetate concentrations remained relatively stable for 15 to 20 hours, during which time ethanol was removed from the blood in a rectilinear fashion. During this time, blood acetate concentrations remained constant at approximately 8 to 9 mg/dL. When blood ethanol concentrations decreased to approximately 80 to 50 mg/dL (= 16 to 10 mM), blood acetate levels began to decline. Blood acetate clearance lagged somewhat behind that of ethanol and appeared to be a function of blood ethanol concentrations. At blood ethanol concentrations higher than 50 to 80 mg/dL, blood acetate levels established a plateau that was observed even when blood ethanol concentrations were as high as 300 to 400 mg/dL. This leveling of the blood acetate concentrations is probably related to the rate of ethanol oxidation. In addition, the height of the acetate plateau of 8 to 9 mg/dL was similar to that under a variety of other situations, one of which occurs after administration of single doses of ethanol in nondependent and nontolerant rats. After a single dose of 3 g of ethanol per kg of body weight and after a short equilibration period, the blood acetate concentration plateau was rapidly established at approximately 8 to 9 mg/dL. The blood ethanol clearance curve followed a straight line, reflecting a rectilinear pattern of ethanol metabolism. A similar pattern of blood ethanol and blood acetate elimination was seen after oral administration of a 6 g/kg dose of ethanol. Under these experimental conditions, blood ethanol levels attained a maximum of approximately 400 mg/dL within 4 hours and blood acetate concentrations were again in the range of 8 to 9 mg/dL.

To summarize, it is evident that the blood acetate curve is similar under a variety of experimental conditions, such as after single doses of ethanol and in ethanol-dependent rats. Our earlier studies indicated a similar pattern of acetate distribution in human subjects during free choice experimental drinking (Majchrowicz, E.: Biochemical Pharmacology of Ethanol, 111-140, New York, Plenum Press, 1975). It is interesting to note that under all these conditions, the blood acetate curve displayed a biphasic character. At blood ethanol concentrations higher than 50 to 80 mg/dL, blood acetate concentrations distributed along a plateau. When blood ethanol concentrations decreased to below 50 to 80 mg/dL, blood acetate appeared to be cleared from the blood in a rectilinear fashion. The plateau in the blood acetate concentration probably reflected the saturation of the enzyme systems that metabolize ethanol. These data suggest that metabolism of ethanol does not vary significantly under the different intoxication conditions used in these studies.

Significance to Biomedical Research and the Program of the Institute:

Although the first two steps in ethanol metabolism, the oxidation of ethanol into acetaldehyde by alcohol dehydrogenase and subsequent oxidation of acetaldehyde by aldehyde dehydrogenase into acetate, have been extensively investigated, relatively little is known about the clearance and metabolism of the ethanol-derived acetate. Since acetate is distributed by the bloodstream throughout the body after being released from the liver, it is important to know the metabolic fate of

this major metabolite of ethanol. It is expected that these data will assist in the better understanding of the molecular mechanisms underlying a variety of effects of ethanol on the intermediary metabolism of extrahepatic tissues and body organs.

Proposed Course:

The project is terminated. A manuscript for publication is being written.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00478-02 LPS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Brain Metabolism and Drugs of Dependence

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

PI:	C. Marietta	Physiologist	LPS, NIAAA
Others:	K. Zbicz	Senior Staff Fellow	LPS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA
	F. Weight	Chief	LPS, NIAAA

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Preclinical Studies

SECTION

N/A

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.6	0.1	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 2-deoxyglucose (2-DG) technique was used to investigate general and localized changes in metabolic activity within the brains of rats exposed to various drugs capable of producing physical dependence. Specifically, we studied the acute effects (one dose), chronic effects (long-term dosing), and the effects of withdrawal from ethanol, phenobarbital, and diazepam. We also investigated the effects of a given dose of diazepam on the ethanol withdrawal syndrome. Similarities were noted in the autoradiographs of animals withdrawing from the drugs studied. Among the similarities noted were a generalized increase in 2-DG uptake, 400-micrometer-wide columns of increased uptake in the frontal sensorimotor cortex, and ovoid areas of increased uptake in the cerebellum. These columns and ovoid areas of increased uptake were no longer visible in the autoradiographs of ethanol-withdrawing rats treated with diazepam before injection of the 2-DG. Differences were also noted in the uptake withdrawal from the three drugs studied. The most striking difference was a localized increase in 2-DG uptake in the dorsal portion of the lateral geniculate in animals withdrawing from phenobarbital and diazepam but not ethanol. Further statistical analysis is needed to identify other similarities and differences in the 2-DG uptake patterns of the withdrawals studied. The comparison of the acute doses of the drugs studied is more complicated. The acute studies of ethanol administration indicate that low doses (0.8 g/kg) increased, moderate doses (1.6 g/kg) decreased, and high doses (3.2 g/kg) showed a mixed response with respect to 2-DG uptake. Acute phenobarbital (100 mg/kg) decreased uptake, while no effect was observed on 2-DG uptake in animals treated acutely with diazepam (5 mg/kg). Further analysis is needed to identify additional differences in the acute animals as well as in the ethanol-withdrawing animals given diazepam.

PROJECT DESCRIPTION:

Investigators:

C. Marietta	Physiologist	LPS, NIAAA
K. Zbicz	Senior Staff Fellow	LPS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
F. Weight	Chief	LPS, NIAAA

Objectives:

The objectives of this project were: (1) to develop dosing schedules that are successful in inducing physical dependence in laboratory rats to ethanol, phenobarbital, 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; and (4) to determine what effects a given dose of diazepam has on the brain metabolism of rats undergoing ethanol withdrawal.

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 was used to determine general and localized changes in brain metabolism. Acute effects were determined by administering the drug under inves- tigation i.p. 30 minutes before the i.v. injection of 2-deoxyglucose. The resulting autoradiographs were analyzed densitometrically.

Major Findings:

We have found statistically significant general and localized increases in [^{14}C]-2-deoxyglucose (2-DG) uptake in animals undergoing withdrawal from ethanol and phenobarbital. In contrast, animals undergoing withdrawal from diazepam show apparent increases in 2-DG uptake when compared to controls; however, these increases were not statistically significant. Similarities and differences were seen in the autoradiographs of animals undergoing withdrawal from ethanol, phenobarbital, and diazepam. Similarities included the appearance of approximately 400-μm-wide columns of increased uptake in the frontal sensorimotor cortex, ovoid areas of increased uptake in the cerebellum, and an increase in 2-DG uptake in several brain nuclei associated with motor function. This is consistent with the preponderance of withdrawal signs that reflect motor functioning, such as hyperactivity, tremors, and spontaneous convulsion. One difference between the ethanol withdrawal and the other two withdrawals occurred in the lateral geniculate. The lateral geniculate showed an increase in uptake in the dorsal portion but not the ventral portion in rats withdrawing from phenobarbital and diazepam but not ethanol.

Acute administration of ethanol produced complex, dose-dependent changes in glucose uptake. At a dose of 0.8 g/kg, 40% of gray structures examined showed an

increase in 2-DG uptake. When given at a dose of 1.6 g/kg, 44% of the gray structures examined showed a decrease in uptake. At 3.2 g/kg, 18% of the gray matter structures examined showed a decrease in uptake while 38% showed an increase in uptake. Ethanol-dependent intoxicated animals also showed an increase in uptake of 2-DG even though the literature documents a decrease in brain O_2 consumption with chronic ethanol administration.

In contrast to acute ethanol administration, acute phenobarbital (100 mg/kg) produced a decrease in 2-DG uptake. This result is similar to other experiments in the literature that indicate a decrease in cerebral O_2 consumption with the administration of phenobarbital.

Acute administration of 5 mg/kg of diazepam produced autoradiographs that appeared similar to controls. This would indicate that diazepam's behavioral effects are not correlated with general changes in brain glucose metabolism. Further densitometric and statistical analysis is necessary to determine if there are any localized changes in 2-DG uptake that might correlate with other known effects of acute diazepam administration.

Diazepam (5 mg/kg) given to ethanol-withdrawing rats yielded autoradiographs in which the columns in the frontal sensorimotor cortex and the ovoid areas of the vermis were no longer visible. These results correlate with an absence of behavioral withdrawal signs in diazepam-treated ethanol-withdrawing animals, indicating that the effect of diazepam on 2-DG uptake during withdrawal correlates with its effects on the behavioral manifestations of withdrawal. Further statistical analysis is necessary to determine if there are additional changes in 2-DG uptake in ethanol-withdrawing animals given diazepam.

Significance to Biomedical Research and the Program of the Institute:

These studies indicate that there are similarities and differences in 2-DG uptake 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) and provide a basis for such investigations. An additional significant observation is that the autoradiographic picture of the brain undergoing ethanol withdrawal correlates with changes in behavior in animals given diazepam, a drug used to treat withdrawal symptoms in man. This suggests the possible use of the 2-DG technique in ethanol-withdrawing rats to screen new drugs for the treatment of ethanol withdrawal.

Proposed Course:

Continued statistical and densitometric analysis will be performed on these projects. A statistical method is being developed to compare, in detail, the similarities and differences in the withdrawal syndromes produced by ethanol, phenobarbital, and diazepam. An investigation into the role of genetics in alcohol metabolism is being planned. This project would compare the response to a given dose of ethanol in rats that have been bred to be alcohol preferring and alcohol avoiding. Several papers are in preparation.

Publications:

Eckardt, M.J., Campbell, G.A., Marietta, C.A., Majchrowicz, E., Wixon, H.N., and Weight, F.F.: Cerebral 2-deoxyglucose uptake in rats during ethanol withdrawal and postwithdrawal. Brain Res. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00462-04 LPS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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

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

PI:	H. Pant	Research Chemist	LPS, NIAAA
Others:	K. Kusano	Research Scientist	LPS, NIAAA
	M. Jeffries	Physiologist	LPS, NIAAA
	E. Majchrowicz	Research Chemist	LPS, NIAAA
	F. Weight	Chief	LPS, NIAAA

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Preclinical Studies

SECTION
N/A

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

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.2	0.7	0.5

CHECK APPROPRIATE BOX(ES)

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

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

Ethanol is known to alter the biochemical and biophysical properties of cellular membranes. In this investigation the mechanisms of ethanol-induced membrane potential changes in rat thymocytes were studied with fluorescence spectrophotometry using membrane potential-sensitive carbocyanine dyes. We analyzed (1) an ethanol-induced fluorescence increase (depolarization) in K-free saline-adapted cells, (2) a prolonged hyperpolarizing potential (fluorescence decrease) induced by adding small amounts of KCl (1-5 mM) in K-+-free saline-adapted cells, and (3) a hyperpolarizing potential induced by the application of the Ca ionophore A23187 in normal saline. The ethanol-induced fluorescence increase in K-free saline solution was completely suppressed by the addition of a small amount of KCl or the K ionophore valinomycin. Hyperpolarizing potentials induced by adding KCl (1-15 mM) in K-free adapted cells were abolished by ouabain; this hyperpolarizing potential was not observed in the absence of Na in the bathing medium. These data indicate that the thymocytes possess a ouabain-sensitive electrogenic Na pump. The in vitro treatment of thymocytes with ethanol (200 mM) for up to 5 hours did not significantly alter this pump potential. However, in thymocytes derived from both ethanol-dependent intoxicated and ethanol-dependent withdrawing rats, the Na pump potential was significantly smaller than that observed in controls. The Ca-dependent hyperpolarization in response to A23187 (50 mM) was not significantly altered by the in vitro treatment of the cells with ethanol (200 mM) for 3 hours. These results suggest that ethanol can depolarize the membrane when the Na pump is inhibited by K-free saline and that alterations in Na pump activity may be associated with ethanol dependence.

PROJECT DESCRIPTION:

Investigators:

H. Pant	Research Chemist	LPS, NIAAA
K. Kusano	Research Scientist	LPS, NIAAA
F. Weight	Chief	LPS, NIAAA
E. Majchrowicz	Research Chemist	LPS, NIAAA
M. Jeffries	Physiologist	LPS, NIAAA

Objectives:

The objective of this investigation was to study the effects of ethanol on the biophysical and biochemical properties of cell membranes. Rat thymocytes were used in this study. Other studies in the laboratory (see Z01AA00477 LPS) have shown that an alteration of lymphocyte number and function in spleen, thymus, and blood is associated with ethanol dependence. We studied the effects of ethanol on (1) membrane potential, (2) electrogenic Na pump activity, and (3) Ca-activated K permeability.

Methods Employed:

The thymus was removed from young male Sprague-Dawley rats and disaggregated mechanically. Dissociated thymocytes were collected by filtration and differential centrifugation. Dissociated thymocytes were suspended in Krebs saline at about 10^5 cells/mL in 2 mL cuvettes and stained with di-S-C_3 (5) (1 μM). The fluorescence intensity of di-S-C_3 (5) was measured by an MPF-44E Perkin-Elmer Fluorescence Spectrometer. The membrane potential of thymocytes in suspension was estimated from a calibration curve, which was obtained by measuring fluorescence intensity change with increasing K concentration in the medium ($[K^+]_o$) in the presence of valinomycin (1 μM), assuming that a change in fluorescence induced by a tenfold change in $[K^+]$ is equivalent to approximately 60 mV in membrane-potential change at 37° C. The zero membrane potential was estimated by adding gramicidin D (25 nM), a channel-forming antibiotic known to make the membrane indiscriminately permeable to monovalent cations. In addition to this, zero membrane potential was confirmed using cells disrupted by sonication in various ionic media. Modified Krebs solutions (i.e., Na^+ free, K^+-substituted [145 mM]; K^+-free, Na^+-free, choline- or Li^+-substituted, etc.) were also prepared in order to examine thymocyte responses in these media. Ethanol-dependent intoxicated rats and ethanol-dependent withdrawing rats were prepared using the method of Majchrowicz (Psychopharmacologia 43:245, 1975). All experiments were carried out at 37° C.

Major Findings:

Ethanol-induced fluorescence increase in K^+-free saline-adapted thymocytes. Ethanol concentrations as high as 300 mM did not induce detectable fluorescence change in thymocytes suspended in normal Krebs solution containing 5 mM K^+. On the other hand, the application of 40 mM ethanol induced a detectable fluorescence increase (depolarization) when cells were suspended in K^+-free Krebs saline. This ethanol-induced fluorescence increase in K^+-free medium was dependent on ethanol concentration. The ethanol-induced fluorescence increase in the K^+-free

medium was completely suppressed by adding KCl (1-5 mM) or 1 μM valinomycin. When valinomycin was added to K^+-free saline-adapted cells, a significant decrease in fluorescence (membrane hyperpolarization) was observed. Application of 5 mM KCl to this preparation always produced an increase in fluorescence. Further increase in K^+ in the medium produced further increase in fluorescence. The thymocyte membrane potential in normal Krebs saline was -55 ± 5 mV and that in K^+-free saline was -32 ± 5 mV. Valinomycin in the K^+-free medium hyperpolarized the thymocyte membrane to about -89 ± 5 mV.

Prolonged hyperpolarizing potentials induced by adding small amounts of KCl (5 or 15 mM) in K^+-free saline-adapted cells. Application of 5 to 15 mM KCl to K^+-free saline-adapted cells induced prolonged hyperpolarization of about 26 mV. This K^+-induced hyperpolarization was completely suppressed by the metabolic inhibitor ouabain. Application of 5 mM KCl to the cell suspension adapted in Na^+-free (choline- or Li^+-substituted) and K^+-free bathing media induced either no change in membrane potential or a small depolarization. These data suggest that the prolonged hyperpolarizing potentials induced by adding a small amount of KCl in K^+-free saline-adapted cells are due to the reactivation of the electrogenic Na^+ pump. Electrogenic Na^+ pump activity in other cell types is known to be inhibited by K^+-free medium. It is also known that when intracellular Na^+ concentration is lowered (as expected in Na^+-free media), the electrogenic Na^+ pump is not operative. Moreover, the Na^+ pump does not extrude Li^+. Indeed, the K-activated hyperpolarization was not observed in Na^+-free choline-substituted medium. Additionally, the K-activated hyperpolarization was not observed when extracellular Na^+ was replaced with Li^+. *In vitro* treatments of thymocytes in K^+-free medium with ethanol (concentrations of 50 mM for 90 minutes, 100 mM for 60 minutes, and 300 mM for 30 minutes) did not produce significant change in the electrogenic Na^+ pump potential activated by adding 15 mM KCl to the K^+-free medium. Electrogenic Na^+ pump activities in thymocytes derived from control animals, ethanol-dependent intoxicated rats, and ethanol-dependent withdrawing rats were compared by measuring the amplitude of the 15 mM KCl-induced hyperpolarization in K^+-free adapted cells. The K-induced hyperpolarization was the largest in thymocytes derived from control rats, followed by ethanol-dependent withdrawing rats, then ethanol-dependent intoxicated rats; the average hyperpolarizations were 26 mV, 18 mV, and 12 mV, respectively.

Hyperpolarizing potentials induced by the application of the calcium ionophore, A23187. Application of the calcium ionophore, A23187 (5×10^{-8} M) to thymocytes in normal Krebs solution induced a $(Ca^{2+})_o$-dependent hyperpolarization. In low Ca^{2+} media this hyperpolarization was diminished while in higher Ca^{2+} concentrations it was increased, suggesting that the hyperpolarizing potential is due to calcium-activated K^+ permeability. The *in vitro* application of ethanol at the concentration of 100 mM for 3 hours did not suppress this hyperpolarization.

Significance to Biomedical Research and the Program of the Institute:

The incubation of thymocytes with ethanol produced a depolarization of cells in K-free medium but did not appear to affect Ca^{2+}-activated K^+ permeability or electrogenic Na^+ pump activity. However, the activity of the electrogenic Na^+ pump appeared to be reduced in thymocytes derived from ethanol-dependent animals compared to control animals.

The significance of the project lies in the observation that ethanol alters membrane mechanisms in thymocytes. The analysis of the action of ethanol on these mechanisms holds the promise of increasing our understanding of the cellular basis of ethanol actions.

Proposed Course:

The acute and chronic actions of ethanol on membrane function will be investigated more extensively.

Publication:

Virmani, M., Majchrowicz, E., Swenberg, C.E., Gangola, P., and Pant, H.C.: Alteration in calcium-binding activity in synaptosomal membranes from rat brains in association with physical dependence upon ethanol. Brain Res. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00472-03 LPS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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)*

PI:	F. Weight	Chief	LPS, NIAAA
Others:	C. Marietta	Physiologist	LPS, NIAAA
	E. Majchrowicz	Research Chemist	LPS, NIAAA
	M. Eckardt	Section Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*

Department of Rickettsial Diseases, Walter Reed Army Institute of Research (T.R. Jerrells); Department of Hematology, Walter Reed Army Institute of Research (R.C. Meagher).

LAB/BRANCH

Laboratory of Preclinical Studies

SECTION

N/A

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.6	0.5	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

Adrenalectomized and nonadrenalectomized Sprague-Dawley rats were addicted to alcohol using a liquid diet supplemented with ethanol by intubation. The rats were sacrificed at various times after ethanol treatment and blood, spleen, and thymus were examined for numbers of lymphocytes and the ability of the lymphocytes to respond to nonspecific T-cell and B-cell mitogens (cell proliferation stimulators). Animals treated with ethanol by inhalation were also tested. Lewis and Sprague-Dawley rats were immunized with sheep red blood cells (SRBC) or TNP-ficol before ethanol treatment, and their ability to respond to an immunization by producing antibodies to the SRBC or TNP-ficol during the period of ethanol administration was tested. Bone marrow cells were also examined to determine the effects of ethanol on the erythroid (red) and myeloid (white) cell progenitors. Both Lewis and Sprague-Dawley rats showed an impaired ability to react to SRBC immunization but not TNP-ficol immunization while being treated with ethanol. Serum corticosterone levels were determined in adrenalectomized as well as normal animals. Animals treated with ethanol showed a decrease in lymphocyte cell numbers in the peripheral blood, spleen, and thymus and an impaired ability of the lymphocytes to respond to nonspecific T-cell and B-cell mitogens. Animals treated with ethanol by inhalation showed a decrease in cell numbers but retained the ability to respond to nonspecific mitogens. Corticosterone levels were increased in ethanol-treated animals. Two corticosterone peaks were seen--one after 2 days of ethanol administration and the other on the day of withdrawal. Seven days after the end of ethanol treatment the rat lymphocytes approached control levels in cell numbers and function. Ethanol treatment, whether by intubation or inhalation, caused a decrease in marrow cellularity and a preferential decrease in colony growth in the erythroid cell line. Further investigations of possible contributing mechanisms of alcohol-induced changes in the immune system are in progress.

PROJECT DESCRIPTION:

Investigators:

F. Weight	Chief	LPS, NIAAA
T. Jerrells	Immunologist	DRD, WRAIR
C. Marietta	Physiologist	LPS, NIAAA
E. Majchrowicz	Research Chemist	LPS, NIAAA
M. Eckardt	Section Chief	LCS, NIAAA
R. Meagher	Research Psysiologist	DH, WRAIR

Objectives:

The objectives of this study were: (1) to determine the effects of alcohol on the immune system in an animal model of alcohol dependence that is consistent and well delineated; (2) to determine the mechanism by which the effects occur; and (3) to compare the effects of alcohol on the immune system using different animal models of alcohol dependence.

Methods Employed:

Male Sprague-Dawley and Lewis rats were made dependent upon ethanol using the intubation technique of Dr. Majchrowicz. Animals were sacrificed daily during the period of intubation their spleens and thymuses removed, and peripheral blood samples collected and analyzed. Animals were also studied 1, 3, 5, and 7 days after the termination of ethanol treatment. Thymus glands were disaggregated and the number of lymphocytes determined. Spleens were also disaggregated, and lymphocytes extracted, counted, and cultured in the presence of nonspecific T-cell mitogens (Con A and PHA) and B-cell mitogens (STM). The ability of the spleen lymphocytes to divide in response to the mitogens was tested using ^{3}H-thymidine in a pulse and harvest experiment. The production of IL2 in response to Con A stimulation was also determined. Adrenalectomized and nonadrenalectomized rats were immunized before the start of ethanol treatment as well as at various times during treatment with sheep red blood cells (SRBC) and TNP-ficol. Spleens were obtained at various times after ethanol treatment, disaggregated, mixed with SRBC or TNP-labeled SRBC (depending upon the agent used for the immunization) and complement, and placed into a Cunningham chamber for a 1-hour incubation. Plaques formed in the lawn of sheep cells were counted. Corticosterone levels were determined by means of radioimmunoassay. Cells from spleen, thymus, and peripheral blood were isolated and stained with fluorescent-labeled monoclonal antibodies to determine the proportion of cells of each subset present (at various times during and after ethanol treatment) using a fluorescence-activated cell sorter. Bone marrow cells from ethanol-treated rats were cultured in methyl-cellulose for 2 or 7 days in the presence of saturating concentrations of either erythropoietin or colony-stimulating factor. Colonies of 8 or more benzedine-positive cells were scored as CFU-E and colonies of 40 or more Wright-stained cells were scored as CFU-GM. In addition, the proliferation studies and bone marrow studies were repeated using male Sprague-Dawley rats treated with ethanol by inhalation.

Major Findings:

Spleen, thymus, and peripheral blood lymphocyte numbers decreased after ethanol treatment, reaching their lowest point after 4 days of treatment. Cell numbers gradually rose following cessation of ethanol treatment and approached control levels 7 days after the end of alcohol treatment. Spleen and peripheral blood lymphocytes showed a decreased ability to respond to Con A, PHA, and STM. The ability of the lymphocytes to respond to mitogen stimulation slowly returned toward control level, taking 7 days after the end of ethanol administration.

The ability of the animals to respond to an immunization with SRBC (a T-cell-dependent antigen) also decreased during ethanol administration, with the lowest point being reached after 4 days of ethanol treatment. Adrenalectomized rats also showed the decreased ability to respond to SRBC immunization. Because there was no change in the ability of adrenalectomized and nonadrenalectomized animals to respond to a TNP-ficol immunization (a T-cell-independent antigen), the effect of ethanol appears to be at the T-cell level. Cell sorter studies are in progress to determine which subset of T-cells, if any, is preferentially affected by ethanol.

IL2 is necessary for lymphocytes to respond to a mitogen by proliferation. The results of the IL2 determinations require further analysis.

Examination of bone marrow from ethanol-treated Sprague-Dawley rats revealed decreased cellularity and 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.

Corticosterone levels were measured because of indications in the literature that increased corticosteroid levels could cause a decrease in lymphocytes, especially in the thymus. Corticosterone levels varied throughout the experiment in both ethanol-treated and control animals. Both ethanol-treated and control animals showed a peak of corticosterone 2 days after beginning intubation, although the ethanol-treated group was significantly elevated (p 0.1). The ethanol-treated animals showed another peak during withdrawas, which was not seen in the control group.

Results similar to those observed when rats were treated with ethanol by intubation were noted in animals treated with ethanol by inhalation. One difference was noted in that although spleen lymphocyte numbers were decreased in the inhalation-treated group, their ability to proliferate in response to nonspecific mitogens (Con A, STM) remained unimpaired. A similar response to ethanol was seen in the bone marrow of animals treated by inhalation.

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, and the function of those remaining lymphocytes is impaired in animals treated with doses of ethanol sufficient to produce dependence. Because lymphocytes are involved in protecting the body from various infectious diseases, these results provide a basis for

investigating the mechanisms involved in the increased risk of alcoholics to infectious diseases.

Proposed Course:

Papers are being prepared that discuss the effects of ethanol on the immune system. Questions to be investigated in detail include: (1) which lymphocyte subsets are disappearing; and (2) where the lymphocytes are going. Further investigations on the mechanism of lymphocyte disappearance are planned.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00476-02 LPS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Neurobiological Correlates of Ethanol Intoxication and Dependence

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

PI:	E. Majchrowicz	Research Chemist	LPS, NIAAA
Others:	M. Kwast	Visiting Fellow	LPS, NIAAA
	G. Bone	Medical Staff Fellow	LCS, NIAAA
	B. Adinoff	Medical Staff Fellow	LCS, NIAAA
	P. Martin	Visiting Scientist	LCS, NIAAA
	H. Moss	Medical Staff Fellow	LCS, NIAAA
	J. Impeduglia	Visiting Fellow	LCS, NIAAA
	M. Linnoila	Chief	LCS, NIAAA

COOPERATING UNITS *(if any)*

University of Tennessee Memorial Research Center & Hospital, Knoxville, TN (R.C. Switzer); Clinical Psychobiology Br, NIMH (L. Tamarkin); Biological Psychiatry Br, NIMH (E. Tamborska, P. Marangos); Clinical Neurosci Br, NIMH (S. Paul)

LAB/BRANCH

Laboratory of Preclinical Studies

SECTION

N/A

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
0.8	0.8	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) Minors

☐ (a2) Interviews

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

The neurobiological correlates of alcohol intoxication and addiction in rats rendered physically dependent upon ethanol were investigated in this project. Our findings during this study included the following. "Peripheral type" benzodiazepine binding sites were possibly more relevant than "central type" receptors in regard to the neurochemical consequences of ethanol dependency. (With utilization of the radioreceptor binding technique, 20-50% increases in the binding of [H-3]-RO5-4864 (a "peripheral type" ligand) to brain membranes derived from the rat cerebral cortex, cerebellum, and hippocampus were observed in ethanol-dependent rats.) These increases persisted for 5 to 7 days following withdrawal from ethanol. In all the brain areas examined, no changes were observed in the "central type" benzodiazepine receptor. This project has been completed.

PROJECT DESCRIPTION:

Investigators:

E. Majchrowicz	Research Chemist	LPS, NIAAA
M. Kwast	Visiting Fellow	LPS, NIAAA
R. Switzer	Research Scientist	U.T.M.R.C. & Hospital
G. Bone	Medical Staff Fellow	LCS, NIAAA
B. Adinoff	Medical Staff Fellow	LCS, NIAAA
P. Martin	Visiting Scientist	LCS, NIAAA
H. Moss	Medical Staff Fellow	LCS, NIAAA
J. Impeduglia	Visiting Fellow	LCS, NIAAA
M. Linnoila	Chief	LCS, NIAAA
L. Tamarkin	Research Biologist	CPB, NIMH
E. Tamborska	Visiting Fellow	Unit Neurochem, BPB, NIMH
P. Marangos	Section Chief	Unit Neurochem, BPB, NIMH
S. Paul	Chief	CNB,NIMH

Objectives:

Consumption of alcoholic beverages is associated with a variety of effects on the peripheral and central nervous system depending on the dose and duration of treatment. Ethanol and other anesthetics are thought to affect the nervous system primarily by direct action on excitable membranes. Ethanol treatment also induces a number of alterations in the activity of several neurotransmitters in the brain that may relate to membrane fluidity changes. Noradrenergic and some cholinergic neurons are activated by ethanol while other cholinergic, serotonergic, and GABAergic neurons are depressed. The disruption of neuronal transmission could modify the properties of these receptors with which the transmitters interact. Thus, ethanol has been reported to affect dopamine receptors, glutamate receptors, adrenergic receptors, and GABA receptors. Studies of central nervous system (CNS) benzodiazepine receptors in rats and mice rendered physically dependent and tolerent to ethanol were also conducted. However, the results obtained were not consistent and seemed to depend on the experimental conditions of ethanol treatment and the kind of benzodiazepine receptor-ligands employed. Subsequently, increases, decreases, and no changes in benzodiazepine receptor binding have been reported.

Benzodiazepines (BZD) and their analogs have been shown to bind to pharmacologically specific sites in the central nervous system (CNS). Shortly after the discovery of BZD binding sites in the CNS, specific, high-affinity [H^3]-diazepam binding was also demonstrated in peripheral organs such as the kidney, lung, liver, and other peripheral tissue. The presence of these so-called "peripheral type" BZD binding sites in the brain has recently been demonstrated using the specific ligand [H^3]-RO 5-4864. The "central type" and "peripheral type" binding sites in the brain are quite distinct in terms of regional distribution, cellular localization, GABAergic effects, and behavioral properties. Although the role of the "peripheral type" benzodiazepine binding site is unclear, recent studies suggest proconvulsant action of "peripheral type" agonists. A preliminary

study indicates that chronic ethanol administration increases the number of "peripheral type" benzodiazepam receptors in mouse brains. In the present report, we confirm and extend these findings in the rat by showing the brain regional distribution of these effects and their time course.

Physical dependence upon ethanol is associated with profound changes in CNS neurotransmission, including an elevation in catecholamines. A daily rhythm exists for the pineal production of melatonin, which is driven by a central circadian oscillator and requires beta-adrenergic stimulation to initiate melatonin biosynthesis. Thus, the diurnal change in pineal melatonin is a useful model with which to explore the effect of the ethanol withdrawal syndrome on a well-characterized neuroendocrine biological rhythm.

Long-term alcohol consumption in chronic alcoholics also results in several neuropathological changes. These changes include cortical atrophy (more marked in the frontal lobes), ventricular enlargement, cell loss, architectural disruption of cortical laminae, and proliferation of glial cells. Cortical atrophy in combination with degeneration of corpus callosum has been described in cases of the Marchiafava-Bignani syndrome. It is generally assumed that these lesions are a direct consequence of ethanol toxicity rather than dietary changes or the metabolism of ethanol in the brain.

This project, using our rat model of physical dependence upon ethanol, has been developed along the five lines of correlative investigations: (1) cerebral binding sites in ethanol-dependent rats, (2) benzodiazepine antagonist R015-1788 and the ethanol withdrawal syndrome, (3) pineal melatonin during ethanol intoxication and withdrawal, (4) elevated blood cholesterol levels during induction and development of ethanol dependency in rats, and (5) degenerated neuronal cell elements in the brains of ethanol-treated rats.

Methods Employed:

Physical dependence on ethanol was induced in male Sprague-Dawley rats by intragastric administration of ethanol (20%, w/v in Sustacal) for 4 days as previously described (Majchrowicz, E.: Psychopharmacologia 43:245, 1975). The following four groups of experimental animals were used.

(1) Single dose group. Rats received a single dose (5 g/kg) of ethanol 4 hours before decapitation.

(2) Prodromal phase group (dependent intoxicated). Rats were rendered physically dependent upon ethanol and decapitated while still intoxicated. Their blood ethanol levels ranged from 380 to 510 mg/dL.

(3) Withdrawal syndrome group (dependent withdrawing). Rats were studied 4 to 6 hours after the onset of overt signs and responses of the ethanol withdrawal syndrome, and 1, 2, 3, 4, and 7 days after withdrawal from ethanol.

(4) Control group. Rats received Sustacal in a pair-matched fashion identical to those receiving ethanol.

All brain areas studied (cerebral cortex, cerebellum, hippocampus, and olfactory bulb) and the kidney were dissected immediately after decapitation of rats and stored at -80° C. Protein determinations were performed using a Bio-Rad assay kit. Before use, the tissues were thawed and homogenized in 25 (cerebral cortex, cerebellum, and hippocampus), 100 (olfactory bulb), or 50 (kidney) volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using Brinkman Polytron (setting 5, 20 seconds). The homogenates were centrifuged at 30,000 xg for 20 minutes. The resulting pellets were washed twice.

Binding assays. Binding assays for both [^{3}H]-BCCE (24 Ci/mM, Amersham) and [^{3}H]-RO 5-4864 (76.5 Ci/mM, New England Nuclear) were performed using the previously described procedures. Tissue homogenates containing 0.15-0.5 mg of membrane protein per assay were incubated with [^{3}H]-BCCE (1.04 nM) ("central type" BZD receptor-ligand) or [^{3}H]-RO 5-4864 (1.3 nM) ("Peripheral type" BZD receptor-ligand) in 50 mM Tris-HCl buffer with a final volume of 0.5 mL. Incubations were carried out at 0° (ice water bath) for 30 minutes, and each assay was terminated by vacuum filtration on Whatman GF-B filters followed by three 5 mL washes with ice-cold buffer. The filters were air-dried and mixed with 10 mL of Beckman Ready-Solv MP. In all cases, 5 μM unlabeled diazepam (Roche, Nutley, N. J.) was used to determine nonspecific binding. Nonspecific binding was about 10-15% and 10-40% of the total binding of [^{3}H]-BCCE or [^{3}H]-RO 5-4864, respectively.

Neuronal cell degeneration. Rats were examined on the day of withdrawal, after the development of overt signs and reactions of the ethanol withdrawal syndrome, or at designated time intervals. Animals were sacrificed by intracardial perfusion with paraformaldehyde while under heavy pentobarbital anesthesia. Brains were removed 1 or more days after perfusion, stripped of their pia membranes, and embedded in a gelatin-sucrose matrix for freeze-sectioning. According to the procedure of de Olmos (1981), sections were incubated in a cupric-silver nitrate mixture for 3 to 4 days before development with silver diamine and reducer solutions. Adjacent sections were silver stained for Nissil substance with thionine.

Major Findings:

Cerebral binding sites. The binding to both central and peripheral BZD receptors was studied in various areas of the brain and kidney at different times after single doses of ethanol and at various stages of the induction and decay of ethanol dependency in rats.

In comparison to the control rats the [^{3}H]-RO 5-4864 binding was observed to be significantly higher in prodromal rats (dependent intoxicated) in the cerebral cortex and cerebellum by 26 $\pm$ 5% ($p < 0.01$) and 35 $\pm$ 6% ($p < 0.01$), respectively. Binding of [^{3}H]-RO 5-4864 was also observed to be significantly increased on days 1, 2, and 3 after ethanol withdrawal by 37 $\pm$ 5% ($p < 0.001$), 53 $\pm$ 5% ($p < 0.001$), and 34 $\pm$ 4% (p 0.05), respectively in the cerebral cortex; 28.4 $\pm$ 8% ($p < 0.05$), 36 $\pm$ 8% ($p < 0.05$), and 43 $\pm$ 6% ($p < 0.001$), respectively in the cerebellum; and 53 $\pm$ 10% ($p < 0.01$), 50 $\pm$ 8% ($p < 0.01$), and 42 $\pm$ 9% (p 0.01), respectively in the hippocampus. However, no significant changes in [^{3}H]-RO 5-4864 binding to cerebral membranes were observed on days 4 and 7 following ethanol withdrawal. Binding of [^{3}H]-RO 5-4864 also increased significantly in the cerebral cortex, cerebellum, and hippocampus after 2 and 3 days of ethanol treatment and after the

initiation of the induction period. No concomitant changes in [^{3}H]-RO 5-4864 binding to either the kidney or olfactory bulb membranes were observed. No significant changes in [^{3}H]-BCCE bindings were observed in any of the brain regions studied during different phases of ethanol dependence. No significant changes in [^{3}H]-RO 5-4864 or [^{3}H]- BCCE binding to all central and peripheral tissues studied were observed after a single dose of ethanol.

The observed number and affinity of "central type" BZD receptors, using [^{3}H]-BCCE as a ligand, was not changed in any of the brain areas studied. These results agree with previous studies where similar findings were reported using the benzodiazepine antagonist [^{3}H]-BCCE as ligand after ethanol liquid diet treatment of 8 days in mice, or [^{3}H]-diazepam for BZD agonists, or [^{3}H]-flunitrazepam after ethanol treatment of up to 3 weeks in mice and rats. However, a decrease in "central type" BZD receptor density was reported after 6 days of ethanol treatment, but this effect was only seen after solubilization of the membrane fraction with detergent Triton X-100. On the other hand, a decrease in BZD receptors was reported after treatment with ethanol for 7 months, while BZD receptor affinity was increased.

Increased [^{3}H]-RO 5-4864 binding sites were observed from 2-3 days after the initiation of ethanol treatment to 6-7 days after ethanol withdrawal. The return of binding to normal levels is, therefore, closely correlated with the disappearance of the withdrawal syndrome. When our results are viewed in relationship to the development of ethanol tolerance, it is possible to suggest that the increase in [^{3}H]-RO 5-4864 binding sites is more related to the development of tolerance than to physical dependence on ethanol.

Benzodiazepine antagonist RO15-1788 (RO) and the ethanol withdrawal syndrome. In order to explore the role of the benzodiazepine-GABA-chloride receptor-ionophore complex in ethanol-benzodiazepine interactions, we administered the central benzodiazepine receptor antagonist, RO15-1788 (RO), to rats displaying overt signs of the ethanol withdrawal syndrome. At low doses, RO is a potent antagonist of benzodiazepine without intrinsic activity of its own. Ethanol-dependent rats undergoing the ethanol withdrawal syndrome were randomly divided into the following four groups: (1) vehicle, (2) RO (5 mg/kg), (3) diazepam (5 mg/kg), and (4) RO and diazepam. Diazepam abolished the signs of ethanol withdrawal syndrome. Neither vehicle nor RO had a significant effect on withdrawal reactions. RO did not significantly antagonize the ameliorating effects of diazepam. These results suggest the two following points: (1) that benzodiazepines do not decrease the severity of ethanol withdrawal symptoms by blocking the effects of an endogenous benzodiazepine "inverse agonist," and that (2) either the central benzodiazepine-GABA-chloride ionophore-receptor complex is not primarily involved in diazepam's reduction of ethanol withdrawal or alcohol dependence modifies the complex so that the expected antagonism of RO is reduced.

Pineal melatonin during ethanol intoxication and withdrawal. The daytime (1500 hours) samples showed no significant difference in melatonin content among controls, prodromals, and withdrawing rats (x = 26.2 pg/pineal). Pineal serotonin content was consistent in the three groups (x = 217.3 ng/pineal). Pineals from rats sacrificed during the dark period (0300 hours), however, showed a significant

decrease ($p < .005$) in pineal melatonin content in the withdrawing rats ($x = 312.0$ pg/pineal) when compared to both controls ($x = 1323.4$ pg/pineal) and prodromals ($x = 1412.3$ pg/pineal). Mean pineal serotonin content was significantly higher in the withdrawing group (201.7 ng/pineal) than in the controls (72.9 ng/ pineal) ($p < .001$). Therefore, there is a diurnal difference in this neuroendocrine system during overt ethanol withdrawal syndrome. Possible explanations at the level of the pineal gland include a subsensitivity of the pineal beta-receptor or depletion of presynaptic norepinephrine. Alterations in neurotransmitters at the level of the central oscillator, perhaps via sympathetic cholinergic pathways to the suprachiasmatic nuclei, may also be responsible.

Cholesterol. It was found that during the induction period, blood cholesterol levels increased from 50 to 70 mg/dL (day 1) to 160 mg/dL on the withdrawal day (day 5) (Majchrowicz, E.: Pharmacologist 25:180, 1983). The highest blood cholesterol levels were found during the prodromal phase (160 mg/dL) while ethanol was still present in the body. As the overt withdrawal syndrome evolved concurrently with the gradual disappearance of ethanol, the blood cholesterol levels decreased to about 130 mg/dL 8 hours after the onset of the neurologically and behaviorally demonstrable ethanol withdrawal syndrome. These data indicate that the elevation of cholesterol in the synaptosomal plasma membrane is paralleled by a similar increase in the blood cholesterol levels.

Neuronal cell degeneration. Rats sacrificed on the withdrawal day displayed conspicuous argyrophilia in the lateral entorhinal and perirhinal cortex as well as in the ventral hippocampal formation. Argyrophilic cell bodies, dendrites, and axons were seen as early as day 2 of the treatment, but not 1, 2, or 3 weeks following withdrawal. No difference was observed between animals sacrificed on the day and 3 days after withdrawal, suggesting no exacerbation due to withdrawal. Dendrites extending to the pial surface formed broad columns, leaving glomerular-like vacancies between them. Caudally, the argyrophilic cell bodies and dendritic fragments were present in the cortex of the rhinal sulcus and continued into the lateral entorhinal cortex layer II and III, but were not present among the large superficial cells of layer II. In some cases, argyrophilic neurons were found in the ventral, but not dorsal, hippocampus in both the pyramidal and dentate regions. In most cases, the argyrophilic neurons extended rostrally into both the rhinal sulcus and pyriform cortex, even to the level of the olfactory peduncle. In some of the more severe cases, a few argyrophilic neurons were found in the lateral amygdala, a structure with reciprocal connections with the layer affected in the entorhinal cortex.

Significance to Biomedical Research and the Program of the Institute:

The differential response of the "peripheral type" and "central type" benzodiazepine binding sites to ethanol treatment underscores the distinct nature of these two sites and provides further evidence for their distinct functional roles. It is possible that a confounding problem of previous studies dealing with the effect of ethanol on brain benzodiazepine receptors relates to the fact that several of the commonly used ligands are [^{3}H]-diazepam and [^{3}H]-flunitrazepams, each of which acts at both types of binding sites. The good agreement between our results and those of Schomaker et al. indicates that, when specific probes for each site are used, the "peripheral type" site is the relevant one. Further elucidation of the functional role of this site in the brain and the development of its specific

antagonists holds the promise of a better understanding of the etiology of alcohol-induced neurological and behavioral disorder syndromes as well as for developing useful agents for the treatment of withdrawal syndromes and alcoholism.

The highest blood cholesterol levels in the dependent intoxicated (prodromal phase) rats coincide with the marked increase in the cholesterol-to-phospholipid molar ratio in the neuronal cell membrane and the highest changes in membrane fluidity. These changes are not isolated in only one body organ but constitute a general adaptive change in the homeostasis of the entire organism. The data in this study also suggest that the administration of alcohol at maximum tolerable doses for a few days results not only in the induction of tolerance and physical dependence upon ethanol but also in a selective destruction of neuronal cells. This degeneration of neurons occurs in various areas of the cerebral cortex and hippocampus, both of which are associated with mental activity and memory. The molecular mechanisms underlying these morphological changes require further investigation.

Proposed Course:

The project has been completed; however, data are still being analyzed. The results of these studies were presented at the Annual Meeting of the American Society for Neurochemistry. The manuscripts of these studies are being prepared for publication.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00438-06 LPS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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

Ethanol and Protein Phosphorylation

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

PI:	H. Pant	Research Chemist	LPS, NIAAA
Others:	P. Gallant	Senior Staff Fellow	LPS, NIAAA
	M. Virmani	Research Chemist	LPS, NIAAA
	N. Ahmad	Visiting Fellow	LPS, NIAAA
	E. Majchrowicz	Research Chemist	LPS, NIAAA
	F. Weight	Chief	LPS, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Neurochemistry and Neuroimmunology, National Institute of Child Health and Human Development, NIH (H. Gainer)

LAB/BRANCH

Laboratory of Preclinical Studies

SECTION

N/A

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
2.9	2.4	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 phosphorylation of protein has been proposed as one of the molecular mechanisms involved in cellular regulation. The protein kinases catalyze these phosphorylation reactions. In this investigation, properties of protein kinase activity associated with cell cytoskeletal proteins have been studied in both neuronal and nonneuronal tissues. In the squid giant axon the kinase activity associated with neurofilaments phosphorylates the high molecular weight 300 and 220 kilodalton neurofilament proteins. The squid axon neurofilament-associated protein kinase (SANFPK) activity appears to be both cyclic-AMP and calcium independent, and can phosphorylate both casein and histone. The squid axon neurofilament protein kinase utilizes both ATP and GTP in the phosphotransferase reaction. In rat brain the kinase activity is associated with microtubule-associated proteins and phosphorylates them. This kinase is cyclic-AMP dependent and its activity increases with low concentrations (14-60 millimolar) of ethanol. In erythrocyte ghosts isolated from rat red blood cells, the kinase activity is associated with the red blood cell cytoskeletal (spectrin and actin) preparation and the kinase specifically phosphorylates spectrin. This kinase also appears to be both cyclic-AMP and calcium independent. Its activity increases in the presence of low concentrations (10-100 millimolar) of ethanol. The observation that ethanol affects protein kinase activity provides an experimental basis for investigating the effects of ethanol on the structure and function of these cytoskeletal proteins and enzymes.

PROJECT DESCRIPTION:

Investigators:

H. Pant	Research Chemist	LPS, NIAAA
H. Gainer	Chief	LNN, NICHHD
P. Gallant	Senior Staff Fellow	LPS, NIAAA
M. Virmani	Research Chemist	LPS, NIAAA
E. Majchrowicz	Research Chemist	LPS, NIAAA
N. Ahmad	Visiting Fellow	LPS, NIAAA
F. Weight	Chief	LPS, NIAAA

Objectives:

A wide variety of extracellular signals, both inside and outside the nervous system, have been proposed to produce many diverse metabolic and physiological responses by regulating the state of phosphorylation of specific substrate proteins in target tissues. The large number of individual molecular pathways involving protein phosphorylation that have been elucidated in animal tissues supports the view that protein phosphorylation is a final common pathway of paramount importance in the regulation of cell function. Cytoskeletal proteins in the neuronal and nonneuronal system appear to undergo significant posttranslational modifications by phosphorylation and limited proteolysis. Certain neurofilament proteins are known to be highly phosphorylated, but little is known about the endogenous kinase activity that phosphorylates these neurofilament proteins. In this investigation we characterized the protein kinases that phosphorylate the cytoskeletal proteins in different tissue preparations. In addition, we have begun to examine the effect of in vitro addition of ethanol on these kinase activities using specific endogenous and exogenous substrates.

Methods Employed:

Isolation of axoplasm. Axoplasm was isolated from squid giant axons of live squid (Loligo pealii) obtained at the Marine Biological Laboratory, Woods Hole, MA. The animals were maintained for a few hours in laboratory tanks containing running sea water. Dissection of the dorsal giant nerve fiber was performed under running sea water on a dissecting table illuminated from below through a glass window. These axons were about 60 mm in length and 400-600 m in diameter. For convenience in subsequent manipulations, each end of the axon was ligated with thread before excision. Dark-field illumination and a dissecting microscope were used for extensive cleaning of the axon. The cleaned axons were blotted to remove the external sea water and the axoplasm was extruded from the intact living axon in the conventional manner so as to avoid contamination of the axoplasm by sea water. Extruded axoplasm was quickly transferred into Eppendorf tubes on ice containing either 100 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA and 20 mM HEPES pH 7.0 (standard phosphorylation buffer medium), or 400 mM NaF, 1% Triton X-100, and 20 mM HEPES pH 7.0 solution for preparation of neurofilament-enriched cytoskeleton.

Fractionation of axoplasm, and preparation of neurofilament-enriched cytoskeleton. Neurofilaments were enriched from the axoplasm obtained from freshly dissected axons as described above and by differential centrifugation. The

extruded axoplasm was collected in 400 mM NaF, 1% Triton X-100, and 20 mM HEPES pH 7.0 homogenized and vortexed for 5 minutes to make a uniform suspension. This was centrifuged at 20,000 rpm for 10 minutes with a Beckman airfuge. The supernatant was discarded and the remaining pellet was washed three times with 100 mM HEPES pH 7.0 to remove Triton X-100. The final pellet was resuspended in standard phosphorylating buffer. In some experiments where axoplasm supernatant was used, the extruded axoplasm was collected in 100 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, and 20 mM HEPES pH 7.0 (instead of 400 mM NaF, 1% Triton X-100 and 20 mM HEPES pH 7.0) before centrifugation. The integrity of the neurofilaments in this preparation was checked by negative staining and electron microscopy using conventional methods.

Measurement of protein kinase activity. Protein kinase activity was measured by using both endogenous and exogenous substrates in the axoplasm, neurofilament, and stellate ganglion cell supernatant preparations. Samples from each tissue preparation in 0.2 mL volume of 100 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, and 20 mM HEPES pH 7.0 were incubated for 10 minutes at 22°C. The phosphorylation reaction was started by the addition of 10 L of ATP (gamma-^{32}P) (0.2 mM) with a specific activity of 29 Ci/mmol, and the reaction was terminated with 10% TCA (trichloroacetic acid) after 10 minutes.

In order to remove TCA-soluble, radioactive material from the samples, the mixtures were centrifuged at 11,000 g for 10 minutes in a Beckman tabletop centrifuge. After discarding the supernatant, the pellet was washed twice with ethanol to remove the residual TCA and then once with acetone. The pellet was dried and dissolved in 100 µL of 2% SDS and 0.01 NaOH, and heated at 100° C for 1 minute. Aliquots (50 µL) of the samples were transferred to glass vials, and counted in 5 mL of aquasol (New England Nuclear) using a Beckman liquid scintillation counter. The kinase activities in the axoplasm, and neurofilament preparations were measured using histone (1 mg/mL) or casein (1 mg/mL) (as exogenous substrate).

Microtubules and microtubule-associated proteins were isolated by three cycles of assembly/disassembly followed by gel filtration using Bio-gel-A-15m (Vallee et al.: J. Cell Biol. 90:568-576, 1981).

Preparation of erythrocyte ghosts. Erythrocyte ghosts were prepared by the method of Dodge et al. (Arch. Biochem. Biophys. 100:119-130, 1963) using isotonic Tris-HCl (pH 7.6) solution. Spectrin was isolated by the method of Bennett et al. (J. Biol. Chem. 252:2753-2763, 1977). Spectrin and spectrin kinase were further purified by DEAE cellulose acetate-52 and Sephadex G-200 column chromatography. The kinase activity was measured as described above.

Major Findings:

Squid axon neurofilament protein kinase (SANFPK) phosphorylates neurofilament proteins and this protein kinase activity is associated with neurofilament-enriched preparation. SANFPK is both a cyclic-AMP-independent and Ca^{2+}-independent protein kinase.

The substrate specificity of SANFPK was examined using increasing concentrations of casein and histone. The rate of phosphorylation increased with increasing concentrations of casein or histone. A double reciprocal plot of the velocity versus substrate concentration data for casein and histone gave an apparent Km of

1.01 mg/mL for casein and Vmax of 16.1 nmoles of [^{32}P]-phosphate incorporated/μg protein/min; and for histone a Km of 2.14 mg/mL and Vmax of 23.8 nmoles/μg protein/min for SANFPK. This indicates that both exogenous casein and histone are good and essentially equivalent substrates for squid axon neurofilament protein kinase.

The phosphorylation reaction catalyzed by SANFPK was examined at various ATP concentrations in the absence (NFP substrate) and presence of exogenous (casein) substrate. A Vmax of 10 nmoles/μg/min was found for both the endogenous (NFP) and casein substrates. The apparent Km was 25 μM for NFP and 58 μM for casein. Similar experiments were conducted using GTP (gamma-^{32}P) as phosphate donor. The Vmax was 4.2 to 4.5 nmoles/μg/min for both the substrates. The Km was 98.1 μM for NFP, and 129.4 μM for casein. That is, both casein kinase II and SANFPK can utilize either ATP or GTP, with a slight preference for ATP.

The protein kinase activity partially purified from rat brain by one cycle assembly/disassembly procedure was found to be cAMP-dependent. The activity of this kinase was measured using histone as an exogenous substrate. Ethanol (40 mM) caused a 50% increase in this kinase activity. At higher ethanol concentration the activity was decreased. Further characterization of this kinase is in progress.

The erythrocyte ghost spectrin kinase phosphorylates spectrin and band 3 proteins, and its activity is associated with spectrin. This kinase activity is cAMP- and Ca^{2+}-independent and phosphorylates casein and spectrin, but not histone. Spectrin kinase also utilizes both ATP and GTP in phosphotransferase reaction with a slight preference for ATP. Unlike SANFPK, activity of this kinase is stimulated by spermine and inhibited by heparin. Ethanol (10-100 mM) increased spectrin kinase activity 25%-50%. Similar effects were observed with higher alcohols at lower concentration.

Significance to Biomedical Research and the Program of the Institute:

The phosphorylation of proteins has been postulated to be of great significance to biological regulatory processes. The cytoskeletal-associated protein kinase activity is affected by low (pharmacological) concentrations of ethanol. The demonstration that ethanol affects protein kinase activity provides an experimental basis for investigating the effects of ethanol on the structure and function of these cytoskeletal proteins and enzymes. This information also provides an opportunity to test the hypothesis that some of the alterations in nervous system function associated with both acute effects of alcohol and alcohol dependence and withdrawal may be due to altered protein phosphorylation.

Proposed Course:

Future study will try to determine which proteins are phosphorylated and dephosphorylated and what other protein kinase, phosphatase, and inhibitor activities are affected in the presence of ethanol. The correlation between protein phosphorylation and the efficacy of neurotransmitter release in synaptosomes derived from squid optic lobe and rat brain also will be examined.

Publications:

Pant, H.C., and Virmani, M.: Calcium regulation of magnesium-dependent phosphorylation of human erythrocyte ghost spectrin. Physiol. Chem. Phys. Med. NMR 84:283-292, 1984.

Pant, H.C., Majchrowicz, E., and Virmani, M.: Cerebral alteration in calmodulin levels associated with the induction of physical dependence upon ethanol in rats. Brain Res. (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00464-04 LPS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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)*

PI:	H. Pant	Research Chemist	LPS, NIAAA
Others:	M. Virmani	Research Chemist	LPS, NIAAA
	P. Gallant	Senior Staff Fellow	LPS, NIAAA
	U. Pande	Visiting Fellow	LPS, NIAAA
	M. Jeffries	Physiologist	LPS, NIAAA
	F. Weight	Chief	LPS, NIAAA

COOPERATING UNITS *(if any)*
Laboratory of Molecular Biology, Univ. of Wisconsin, Madison, WI (R. Silver)

LAB/BRANCH
Laboratory of Preclinical Studies

SECTION
N/A

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	0.9	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.)*

Alcohol is known to affect developing tissues, evidenced by the fetal alcohol syndrome, but the molecular mechanisms of such effects are not known. In this study we have used sand dollar eggs to investigate the effects of ethanol on early stages of cell division. Video-enhanced microscopy was used to study the effects of ethanol on mitosis of sand dollar eggs. Our preliminary study showed that in the presence of low concentrations (30-50 mM) of ethanol, the immediate effects included paralysis of sperm tail motility and distortion of the embryo fertilization envelope. Later effects included disruption of cytoplasmic organization and inhibition of mitosis. The data suggest that ethanol may affect the ability of a cell to progress through the cell cycle and mitosis. Similar results were obtained when micromolar calcium chloride was injected into these eggs. The studies on sand dollar eggs could provide an experimental model for investigating the mechanism of the effects of ethanol on the early stages of development.

The intracellular calcium concentration in clonal AtT-20/D16 mouse anterior pituitary tumor cells and in rat hepatocytes was measured with the fluorescent calcium indicator Quin 2. A fourfold increase in intracellular calcium was observed in 50 mM KCl Krebs solution compared to 5 mM KCl normal Krebs solution. In normal Krebs solution, ethanol (30 mM) caused a 10% increase in intracellular calcium concentration in rat hepatocytes, but under these conditions AtT-20/D16 cells showed no significant change. These observations suggest that the effects of ethanol on cellular calcium metabolism may vary in different cell types.

PROJECT DESCRIPTION:

Investigators:

H. Pant	Research Chemist	LPS, NIAAA
P. Gallant	Senior Staff Fellow	LPS, NIAAA
U. Pande	Visiting Fellow	LPS, NIAAA
M. Virmani	Research Chemist	LPS, NIAAA
R. Silver	Assoc. Prof.	Univ. Wisc.
F. Weight	Chief	LPS, NIAAA
M. Jeffries	Physiologist	LPS, NIAAA

Objectives:

An increase in free calcium ion (Ca^{2+}) concentration within stimulated cells underlies such important biological processes as muscle contraction, the secretion of transmitters and hormones, the regulation of enzyme activities, and the control of membrane ion permeabilities. The cytosolic calcium concentration of the unstimulated cell is maintained at levels three to four orders of magnitude lower than in the extracellular medium by the extrusion of the ion by Ca^{2+} pumps and exchange mechanisms in the plasma membrane, as well as by uptake by intracellular sequestration sites. Our objectives were to study the effects of ethanol on cell mitosis and cellular calcium metabolism.

Methods Employed:

In order to test the effects of alcohol on cell division and embryonic development, sand dollar embryos were exposed to pharmacological concentrations (30-50 mM) of ethanol and their development was followed with light microscopy. Eggs of the sand dollar Echinarachnius parma were fertilized in artificial sea water (ASW) and washed free of sperm. At 15 minutes after fertilization, eggs were placed in a special observation chamber containing artificial sea water supplemented to 30-100 mM ethanol. Embryos were observed with Nomarski differential interference contrast optics and time-lapse video recording.

The clonal AtT-20/D16 mouse anterior pituitary tumor cells were grown in standard culture medium. Cells (2-4 x 10^6 cells/mL) were loaded with Quin 2 by incubation with the acetoxymethylester derivative of Quin 2 (Quin 2/AM), which was subsequently hydrolyzed and trapped inside the cells. The fluorescence signal was measured from these cells in normal Krebs solution or modified Krebs solution using an MPF-44E spectrofluorometer. A similar procedure was used to load Quin 2 in rat hepatocytes.

Major Findings:

Sand dollar eggs. The immediate effects of ethanol (50 mM) included paralysis of sperm tail motility and a distortion of the embryo's fertilization envelope. Later effects included disruption of cytoplasmic organization. These cells failed to divide.

AtT-20/D16 cells and rat hepatocytes. Quin 2 fluorescence measurements showed that 30 mM ethanol produced a 10% increase in intracellular calcium concentrations in rat hepatocytes in normal Krebs solution. In AtT-20/D16 cells under similar conditions, 30 mM ethanol produced no detectable increase in intracellular calcium levels. In both cells, 50 mM KCl-containing Krebs solution increased intracellular calcium concentration fourfold compared with normal Krebs saline solution.

Significance to Biomedical Research and the Program of the Institute:

The unique role of Ca^{2+} as an activator and regulator of many biological processes has been well recognized. An increase in cytoplasmic Ca^{2+} concentration underlies such important biological processes as the secretion of transmitters and hormones, the regulation of enzyme activities, control of membrane ion permeabilities, and muscle contraction. There are a number of cellular mechanisms that control cytosolic Ca^{2+}. The results of the rat hepatocyte studies indicate that ethanol may affect the cytosolic concentration of ionized Ca^{2+}. Similar results were previously obtained in synaptosomes isolated from rat brain. These observations suggest that ethanol may affect the mechanisms regulating cellular Ca^{2+} metabolism. The studies on sand dollar eggs could provide an experimental basis for investigation of the effects of ethanol on the early stages of development.

Proposed Course:

Continuation of the project will be directed toward elucidating the ethanol/calcium relationship in cellular systems derived from alcohol-treated animals and cell lines in tissue culture.

Publications:

Pande, U., and Pant, H.C.: Effect of ethanol on calcium metabolism in rat erythrocyte ghosts. Physiol. Chem. Phys. and Med. NMR 16:463-467, 1984.

Pande, U., and Pant, H.C.: Evaluation of calcium-arsenazo III stoichiometry in the presence and absence of EGTA at various pH. Analytical Letters (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00474-02 LPS

PERIOD COVERED
October 1, 1984 to September 30, 1985

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

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

PI:	H. Pant	Research Chemist	LPS, NIAAA
Other:	P. Gallant	Senior Staff Fellow	LPS, NIAAA

COOPERATING UNITS *(if any)*
Laboratory of Neurochemistry and Neuroimmunology, National Institute of Child Health and Human Development, NIH (H. Gainer).

LAB/BRANCH
Laboratory of Preclinical Studies

SECTION
N/A

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.60	0.60	

CHECK APPROPRIATE BOX(ES)

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

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

Brain damage occurs in over 60% of chronic alcoholics. The causes and pathophysiology of this damage, however, are poorly understood. The general goal of our research has been to evaluate the factors that lead to alcohol-induced degeneration of the nervous system in experimental animals. It is generally agreed that at least one type of alcohol-induced brain damage, Wernicke-Korsakoff's encephalopathy, is due to alcohol-induced thiamine deficiency. We have therefore studied the effect of thiamine deficiency on rat brain. Thiamine-deficient rats exhibited a breakdown of the blood brain barrier as evidenced by small hemorrhages and an accumulation of blood proteins in the brain. Biochemically these brains demonstrated an accumulation of proteins normally found in the blood; for example, albumin, fibrinogin, and hemoglobin. The breakdown of the blood brain barrier may lead to some of the irreversible brain damage and neurological deficits associated with thiamine deficiency.

The role of calcium ions in the degeneration of nervous tissue is being investigated in the squid giant axon. In the squid axon we have identified a number of immunoreactive degradation products after calcium-activated proteolysis. The results suggest that an elevation of cytosolic ionized calcium may result in a breakdown of proteins.

PROJECT DESCRIPTION:

Investigators:

H. Pant	Research Chemist	LPS, NIAAA
P. Gallant	Senior Staff Fellow	LPS, NIAAA
H. Gainer	Chief	LNN, NICHHD

Objectives:

Brain damage occurs in over 60% of chronic alcoholics. The causes and pathophysiology of this damage, however, are poorly understood. The general goal of this project has been to evaluate the factors that lead to alcohol-induced degeneration of the nervous system in experimental animals. It is generally agreed that at least one type of alcohol-induced brain damage, Wernicke-Korsakoff's encephalopathy, is due to alcohol-induced thiamine deficiency. We are therefore examining the mechanisms by which thiamine deficiency leads to this degeneration. Other types of neuronal damage, however, appear not to be related to nutritional deficiences, but may instead be due to the direct effects of alcohol on nerve cells. Biochemically, different types of degeneration may have a common basis. We are testing the hypothesis that alcohol causes an increased intracellular Ca^{2+}, which then activates catabolic enzymes leading to neuronal degeneration.

Methods Employed:

To induce thiamine deficiency, male Sprague-Dawley rats were fed a thiamine-deficient diet only or were fed a thiamine-deficient diet and received an injection of 1 mg/kg/day pyrithiamine (a thiamine antagonist). After developing neurological symptoms of thiamine deficiency, animals were anesthetized and perfused with saline, and their brains were sectioned and photographed, or the brains were removed and the brain proteins were extracted by homogenization in hypotonic buffer. SDS was then added to the supernatant and the proteins were analyzed by SDS polyacrylamide gel electrophoresis (PAGE). Albumin was quantified immunochemically. To study calcium-activited proteolysis in the squid giant axon, axoplasm was extruded and exposed to various concentrations of Ca^{2+}. The proteins were then separated by PAGE, and stained by Coomassie Brilliant Blue, silver, or with HRP-labeled antibodies.

Major Findings:

The breakdown of the blood brain barrier to plasma proteins was studied in thiamine-deficient rats. In rats made deficient with a thiamine-deficient diet plus 1 mg/kg/day pyrithiamine, clear neurological symptoms appeared 11 to 15 days after the initial pyrithiamine injection. Symptoms included ataxia, weakness, opisthotonic convulsions, and eventually loss of righting reflex and death. Macroscopically the brains of the opisthotonic animals exhibited small (petechial) hemorrhages. Biochemically these brains demonstrated an accumulation of proteins normally found in the blood; for example, albumin, fibrinogin, and hemoglobin. Animals made thiamine deficient by diet alone exhibited the same symptoms except that the symptoms started 40-50 days after the initiation of the thiamine-deficient diet and the animals did not lose their righting reflex before they

died. Also in contrast to the pyrithiamine-treated group, the diet group did not exhibit as many hemorrhages and no accumulation of albumin was detectable in their brains. The massive breakdown of the blood brain barrier characteristic of the pyrithiamine-treated animals is thus not necessary to the production of the initial neurological deficits in thiamine-deficient rats, but may be related to some of the latter symptoms (loss of righting reflex) and perhaps to some of the irreversible damage.

To directly investigate the role of calcium in the degeneration of nervous tissue, calcium-activated proteolysis and its effects on neurofilament structure were studied in the squid giant axon. In this preparation we found that millimolar concentrations of ionized calcium are required to activate proteolysis, that a protease is activated when the axon is metabolically poisoned, and that the proteolysis is prevented if calcium chloride is removed from the outside medium. We identified a number of new calcium-activited proteolysis fragments immunochemically and found that even fresh axons had a small amount of these breakdown products, which suggests that limited calcium-activated proteolysis may occur in healthy nerves.

Significance to Biomedical Research and the Program of the Institute:

Our investigating in rats and the squid giant axon on the mechanisms by which cells become irreversibly damaged after ethanol treatment, nutritional deficiencies, and intracellular calcium ion accumulation provides a basis for investigating ethanol-induced degeneration in nervous tissue. A number of alcohol-induced neuropathies have no clear association with nutritional deficiencies. We are therefore also investigating the possibility that ethanol can lead to neuronal degeneration directly, without inducing nutritional deficiences.

Proposed Course:

We intend to complete and publish our work on the breakdown of the blood brain barrier during thiamine deficiency. We will also study the basic mechanism of calcium-induced proteolysis in the squid axon, including studies to determine if ethanol disturbs intracellular calcium metabolism directly in extruded axoplasm. In addition, we intend to test the hypothesis that alcohol increases intracellular proteolysis in nerve cells by increasing intracellular Ca^{2+} levels.

A paper entitled "Phosphorylation and calcium-activated proteolysis of neurofilament proteins in the squid giant axon" has been submitted for publication.

Publications:

Gallant, P.E., Pant, H.C., and Gainer, H.: Distribution of acid protease activity in squid nervous system. J. Neurochem. 42:590-593, 1984.

Gainer, H., Gallant, P.E., Gould, R., and Pant, H.C.: Biochemistry and metabolism of the squid giant axon. In P. Baker (Ed.). Current Topics in Membranes and Transport. Academic Press, New York, 22:57-90, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00479-02 LPS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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)*

PI:	F. Weight	Chief	LPS, NIAAA
Others:	S. Korn	PRAT Fellow	LPS, NIAAA
	D. Lewis	Staff Fellow	LPS, NIAAA
	C. Rabe	Staff Fellow	LPS, NIAAA

COOPERATING UNITS *(if any)* Dept. Path., Duke U. (G. Campbell); Epil. Ctr., U. Texas, Dallas (H. Doller); Lab. Cell Biol., NIMH (A. Luini); Dept. Pharm., U. Alberta (P. Smith); Dept. Physiol., Creighton U. (J. Wilson); Dept. Anat., Howard U. (J. Wilson); Dept. of Neurobiol., Max-Planck Inst., Frankfurt, West Germany (P. Yavari)

LAB/BRANCH

Laboratory of Preclinical Studies

SECTION

N/A

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.2	3.2	

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.)*

Ethanol has been reported to alter synaptic transmission and neurosecretion; however, the mechanisms involved in these actions are poorly understood. Neurosecretory mechanisms and the actions of ethanol on those mechanisms were studied in cell lines in tissue culture. In the mouse pituitary cell line, AtT-20, somatostatin inhibits both the secretion of ACTH and the cytosolic calcium rise evoked by secretagogues. We studied the effect of somatostatin on membrane calcium current in these cells using the whole-cell patch-clamp technique. Calcium current activated rapidly at potentials positive to -40 mV, peaked in 6-7 ms, and inactivated slowly. Somatostatin (0.01 to 1 micromolar) decreased the calcium current by 30% during voltage steps eliciting maximal current amplitude. The results suggest that the somatostatin-induced inhibition of ACTH secretion may result from the reduction of peak calcium current by somatostatin. Neurosecretory mechanisms were also investigated in the rat chromaffin cell line, PC12. We studied the effect of muscarinic agonists on intracellular calcium, phosphoinositide metabolism, and transmitter release. Addition of the muscarinic agonists methacholine or muscarine caused an increase in intracellular free calcium levels. The increase in intracellular calcium was blocked by atropine; however, removal of extracellular calcium did not block the response. In addition, methacholine increased cellular levels of inositol triphosphate and stimulated the release of norepinephrine. These results indicate that PC12 cells provide a model for investigating the interrelationship between phosphatidylinositol metabolism, intracellular free calcium, and secretion. The effects of ethanol are being tested on the secretory mechanism in these two cell lines. The significance of the project derives from the fact that characterization of synaptic and 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.

PROJECT DESCRIPTION:

Investigators:

F. Weight	Chief	LPS, NIAAA
G. Campbell	Resident	D.P., Duke U.
H. Doller	Asst. Prof.	E.C., U. TX, Dallas
S. Korn	PRAT Fellow	LPS, NIAAA
D. Lewis	Staff Fellow	LPS, NIAAA
A. Luini	Visiting Fellow	LCB, NIMH
C. Rabe	Staff Fellow	LPS, NIAAA
P. Smith	Asst. Prof.	D.P., U. Alberta
J. Wilson	Asst. Prof.	D.P., Creighton U.
J. Wilson	Asst. Prof.	D.A., Howard U.
P. Yavari	Visiting Scientist	D.N., Max-Planck

Objectives:

Ethanol has been reported to alter synaptic transmission and neurosecretion; 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.

Methods Employed:

Previous studies indicate that ethanol can alter both neurotransmitter release and neurosecretion. In other studies in this laboratory, Dr. Pant and coworkers found that ethanol alters 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 now possible to study ion channels or intracellular Ca^{2+} signals in nerve terminals, we have recently begun experiments on two neurosecretory cell lines: (1) the mouse pituitary cell line, AtT-20; and (2) the rat chromaffin cell line, PC12.

AtT-20 cells. Somatostatin inhibits the secretion of ACTH evoked by secretagogues, including corticotropin-releasing factor, vasoactive intestinal peptide, isoproterenol, and forskolin in mouse pituitary tumor AtT-20 cells. In recent studies on AtT-20 cells, Luini *et al*. found that: (1) somatostatin decreases the basal cytosolic Ca^{2+} rise evoked by the above secretagogues; (2) nifedipine reduces basal and secretagogue-stimulated cytosolic Ca^{2+} levels; (3) the effects of somatostatin and nifedipine are not additive; and (4) TEA does not block the effect of somatostatin on cytosolic Ca^{2+}. These results raise the possibility that somatostatin may affect membrane Ca^{2+} conductance. In view of this possibility, we used the patch-clamp method to study the effect of somatostatin on the Ca^{2+} current in these cells.

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. Currents were recorded in an external solution containing (in mM): 150 TEA, 0.8 $MgCl_2$, 5.4 KCl, 10 $CaCl_2$,

10 HEPES (pH 7.4), 45 glucose, 10^{-6} M TTX, and 1 mg/mL albumin with an osmolarity of 340 mOsm. The solution in the patch pipette contained (in mM): 120 CsCl, 11 EGTA, 2 TEA, 2 $MgCl_2$, 10 HEPES (pH 7.4), 4 Mg_2ATP, 20 creatine phosphate, and 50 units/mL creatine kinase with an osmolarity of 318 mOsm. All recordings were at room temperature.

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 have 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 μM Quin 2/AM for 20 minutes, washed, and resuspended at a concentration of 2 x 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 μCi/mL) for 24 hours. To investigate neurotransmitter release, cells were preloaded for 30 minutes with [^{3}H]-norepinephrine (500 nM, 23.1 Ci/mmol).

Major Findings:

AtT-20 cells. 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 Gohms. An inward current activated rapidly at potentials positive to -40 mV, peaked in 6-7 ms, inactivated slowly, was inhibited by 2 mM 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. Somatostatin (10^{-8} to 10^{-6} M) was applied from a micropipette lowered into the external solution near the cell under study. Somatostatin decreased peak I_{Ca} by 30.1 $\pm$ 6.7% (N = 6) during voltage steps eliciting maximum current amplitude (+10 to +15 mV). Within 2 minutes after beginning the somatostatin washout, I_{Ca} returned to the control level. Control solutions of peptide vehicle (acidified bovine serum) diluted with external solution (identical to the peptide dilutions) had no apparent effect on I_{Ca}. The results suggest that the somatostatin-induced inhibition of ACTH secretion may result from the reduction of peak calcium current by somatostatin.

PC12 cells. Quin 2-loaded PC12 cells had a resting free intracellular Ca^{2+} level of approximately 110 nM using the calibration method of Tsien et al. (Nature 295:68, 1982). Addition of muscarinic agonists of the low affinity subtype, M_2, methacholine (100 μM) or muscarine (100 μM), caused a 50 to 60 nM increase in intracellular free Ca^{2+} levels. In contrast, agonists of the M_1 subtype, oxotremorine (1 mM) or McN-A-343-11 (1 mM), had no detectable effect on intracellular Ca^{2+} levels. The increase in intracellular Ca^{2+} produced by methacholine was blocked by atropine (100 nM). However, removal of Ca^{2+} from the extracellular medium did not block the ability of the M_2 agonists to raise intracellular Ca^{2+} levels. This suggested that the increase in intracellular Ca^{2+} might be the result of mobilization of Ca^{2+} from intracellular stores. Consistent with that hypothesis was the observation that methacholine treatment increased cellular levels of inositol triphosphate (IP_3), a metabolite of phosphatidylinositol bisphosphate shown to mobilize Ca^{2+} from intracellular

stores in several other cell types (Nature 312:315, 1984). When cells had been prelabeled with [^{3}H]-inositol, methacholine (200 μM) doubled [^{3}H] IP_3 levels within 30 seconds (control 993 + 71; methacholine 1960 $\pm$ 15 cpm/mg protein). The rate of [^{3}H]-inositol bisphosphate and [^{3}H]-inositol phosphate also increased, but with a slightly slower time course. We also examined the effects of muscarinic receptor activation on secretion. In the absence of other stimuli, methacholine (100 μM) caused a stimulation of catecholamine release from PC12 cells that had been preloaded with [^{3}H]-norepinephrine. Although it is unclear whether the muscarinic activation of secretion was a direct result of Ca^{2+} mobilization, the PC12 cells clearly provide a model system for examining the interrelationship between muscarinically induced phosphatidylinositol metabolism, intracellular free Ca^{2+}, and secretion.

The effect of ethanol on neurosecretory mechanisms in AtT-20 and PC12 cells is 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.

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 depressants such as barbiturates, opiates, and benzodiazepines will be characterized and compared to ethanol. Papers on neurosecretory mechanisms in AtT-20 and PC12 cells are in preparation.

Publications:

Weight, F.F.: Postsynaptic mechanism in long-lasting potentiation of synaptic transmission. In McGaugh, J.L., Lynch, G., and Weinberger, N.M. (Eds.): Neurobiology of Learning and Memory. New York, Guilford Publ., 1984, pp. 491-499.

Campbell, G.A., Eckardt, M.J. and Weight, F.F.: Dopaminergic mechanisms in subthalamic nucleus of rat: Analysis using horseradish peroxidase and microiontophoresis. Brain Res. 333:261-270, 1985.

Doller, H.J., and Weight, F.F.: Perforant pathway evoked long-term potentiation of CA1 neurons in the hippocampal slice preparation. Brain Res. 333:305-310, 1985.

Schofield, G.G., and Weight, F.F.: Single acetylcholine channel currents in sympathetic neurons. Brain Res. 343:200-203, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00480-02 LPS

PERIOD COVERED

October 1, 1984 to September 30, 1985

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)*

PI:	F. Weight	Chief	LPS, NIAAA
Others:	S. Ikeda	Medical Staff Fellow	LPS, NIAAA
	G. Schofield	Visiting Associate	LPS, NIAAA
	K. Zbicz	Senior Staff Fellow	LPS, NIAAA

COOPERATING UNITS *(if any)*

Laboratory of Neurophysiology, NINCDS, NIH (A.B. MacDermott).

LAB/BRANCH

Laboratory of Preclinical Studies

SECTION

N/A

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
3.2	3.2	

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 ionic basis of nerve cell excitability and the actions of ethanol on those mechanisms were investigated using electrophysiological methods. Sodium and calcium currents were characterized in adult mammalian neurons acutely isolated from rat nodose ganglion using the whole cell patch-clamp method. Two types of sodium current were observed. One current was abolished by 3-15 micromolar 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 micromolar TTX, had a slower time course, activated over the potential range -30 to 0 mV, and attained half-maximal conductance at -10 mV. Two calcium current components were observed in some cells, an inactivating component that activated near -60 mV, and a large sustained current that activated near -40 mV. The results indicate that some cells have more than one type of sodium and/or calcium channel. Calcium current was also studied in CA3 pyramidal neurons in hippocampal slice using the single-electrode voltage clamp. The calcium current activated rapidly and gradually decayed with maintained depolarization. Upon return to a more hyperpolarized potential, the calcium current decayed with both a rapid and a slow component. The rapid component was too fast to be followed by the single-electrode clamp. The decay of the slow component could be described by a single exponential function that was dependent upon the potential of the neuron. The decay time course suggests that there are two or more types of calcium channels in these central neurons. The effect of ethanol is being tested on these ion currents. The significance of the project lies in the fact that the identification of the mechanisms involved in nerve cell excitability and the action of ethanol on those mechanisms hold the promise of increasing our understanding of the cellular basis of ethanol's actions in the nervous system.

PROJECT DESCRIPTION:

Investigators:

F. Weight	Chief	LPS, NIAAA
S. Ikeda	Medical Staff Fellow	LPS, NIAAA
A. MacDermott	Senior Staff Fellow	LN, NINCDS
G. Schofield	Visiting Associate	LPS, NIAAA
K. Zbicz	Senior Staff Fellow	LPS, NIAAA

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 two electrophysiological methods: (1) whole cell patch-clamp recording of acutely isolated adult rat nodose ganglion cells; and (2) single-electrode voltage clamp recording of CA3 pyramidal neurons in hippocampal slice.

Patch-clamp recording:

Cell isolation procedure. Single nodose 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 were placed in iced Hank's balanced salt solution (HBSS). The nodose ganglia were removed and placed in 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 hour at 35° C in a 25 cm^2 tissue culture flask that allowed microscopic inspection of the tissue fragments. After incubation, the flask was shaken vigorously, 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 an 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 was 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 (3 KHz-3 dB) (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 μsec/point (Na^+ currents) or 150 μsec/point (Ca^{++} currents). Current traces and current/voltage relationships were corrected for linear leakage current measured from hyperpolarizing command pulses. Periods of 400-450 μsec at the onset and offset of the command pulses have been removed from all current records to remove spurious points due to incomplete capacitive compensation. Test solutions were applied by lowering a large bore (>10 μm) pipette containing the test solution, close to the cell soma. All experiments were conducted at 19 to 23° C.

Single-electrode voltage clamp:

Brain slice preparation. Slices were prepared from the hippocampus, which was rapidly removed from male Hartley guinea pigs (300-600 g). The hippocampus was cooled by immersion in iced artificial cerebrospinal fluid (CSF) and slices (400-450 μm) 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 hour, 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 had the following composition (mM): NaCl 124, KCl 3.2, $CaCl_2$ 2.4, $MgCl_2$ 1.3, $NaHCO_3$ 26, NaH_2PO_4, and 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; Fredrick 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 were 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-sec voltage steps and at 750 Hz when observing faster events during 100-msec steps. The microcomputer was also used to generate the voltage commands.

Major Findings:

Whole cell patch-clamp recording of nodose 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 recordings 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-36 mV, and rate of rise 117 V/sec. Two types of action potentials, short duration and long duration, were observed. 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 μm 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 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 μM 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 μM TTX, had a slower time course, activated over the potential range -30 to 0 mV, and attained half-maximal conductance at -10 mV. In addition, 500 μM Cd^{++} and 5 mM Co^{++} reduced the TTX-insensitive current to 53% and 42% of control, respectively. Inward Ca^{++} current was isolated by ion substitution, and pharmacological agents were identified by a dependence on external Ca^{++}. Cd^{++} (500 μM) and 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 that activated near -60 mV, and a large sustained current that 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.

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 decline in inward current was also observed when tetraethylammonium chloride (124 mM) was substituted for NaCl or Ba^{2+} was substituted for Ca^{2+} in the superfusing solution. In addition, the inward current activated during step depolarizations decreased as the holding potential was made more positive. The inward Ca^{2+} current activated by a depolarizing step decayed with a complex time course upon return to a more hyperpolarized potential. This decay had both rapid and slow components. The rapid component was too fast to be accurately followed by the SEC. The slow component was observed as an inward tail current, the decay of which could be described by a single exponential function. The time constant for decay of this slow component was dependent upon the potential of the neuron, decreasing at more hyperpolarized potentials. 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. The deactivation time course for the inward current suggests that two or more types of Ca^{2+} channels are present in these cells. The effect of ethanol on ion currents in nodose ganglion cells and CA3 pyramidal neurons is currently being tested.

Significance to Biomedical Research and the Program of the Institute:

The psychological and 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 hold the promise of increasing our understanding of the cellular basis of ethanol's actions on 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 benzodiazepines will be characterized and compared to ethanol.

Publications

Zbicz, K.L., and Weight, F.F.: Transient voltage and calcium-dependent outward currents in hippocampal CA3 pyramidal neurons. J. Neurophysiol. 53:1038-1058, 1985.

Annual Report of the Laboratory for Studies of Neuroadaptive Processes
National Institute on Alcohol Abuse and Alcoholism
October 1, 1984 to September 30, 1985
Boris Tabakoff, Ph.D., Acting Chief

Introduction

The Laboratory for Studies of Neuroadaptive Processes was established in 1984 with the goal of investigating the neurochemical mechanisms associated with adaptive responses of the central nervous system (CNS) to ethanol, i.e., the development and expression of functional tolerance and physical dependence. Two approaches have been followed in developing research strategies. First, it was necessary to define initial sites of action for ethanol in the CNS, in order to determine whether chronic ethanol ingestion, which produces tolerance and physical dependence, generates adaptive changes at these sites. A current hypothesis suggests that ethanol exerts its CNS effects via perturbation of neuronal membrane lipids. However, proteins, rather than lipids, represent the important functional moieties of neuronal membranes, although activities of such membrane-bound proteins are influenced by their lipid microenvironments. During the past year, much of our research has focused on elucidation of the acute and chronic effects of ethanol on the activity of membrane-bound proteins involved in synaptic transmission and neuronal function. The results indicate that ethanol acts at specific sites within the neuronal membrane to selectively influence the activity of various proteins, as well as protein-protein interactions. In some instances protein properties appear to be influenced secondarily to changes in membrane lipid characteristics, and in other cases ethanol may directly affect the proteins. Crucial findings are that ethanol has specific sites of action in the CNS, and that adaptation to the acute effects of ethanol can be demonstrated in tolerant animals.

The second approach we have taken to understand neuroadaptive processes is to evaluate the mechanism of action of agents that can modulate ethanol tolerance and/or dependence. We had shown earlier that the neurohypophyseal hormone, arginine vasopressin (AVP), maintains functional ethanol tolerance in animals in which tolerance has been established, even in the absence of further ethanol intake. This action of AVP depends on the presence of intact noradrenergic systems in the brain. Our work in the past year has focused on establishing that AVP acts within the CNS to affect tolerance, and that endogenous hormone is important for maintenance of ethanol tolerance. We have now begun to investigate possible biochemical mechanisms of action of AVP in the brain.

1. Ethanol Effects on Neurotransmitter Receptor-Effector Coupling Processes

An important system for generating intracellular messages in response to hormones or neurotransmitters is the adenylate cyclase complex. The receptor adenylate cyclase system consists of at least three protein components--receptor, guanine nucleotide-binding protein (N), and catalytic unit of the enzyme--all of which interact within the cell membrane. Detailed models for these interactions have been proposed, and within the framework of such models the effects of ethanol can be evaluated. In cerebral cortex, ethanol

acutely increases basal, guanine nucleotide, and isoproterenol- stimulated adenylate cyclase (AC) activity. Ethanol acts at specific sites, including the receptor, N(s), the catalytic unit of AC, and the interaction between N(s) and the catalytic unit. In ethanol-tolerant and dependent animals, the response of cortical AC to guanine nucleotides and isoproterenol was reduced, suggesting that an adaptation to the effects of ethanol had occurred. Similar results were found when the beta-adrenergic receptor-AC system in pineal gland was examined. In contrast, ethanol (acutely, in vitro, or after chronic in vivo administration) had no effect on the inhibition of striatal AC activity by opiates. These results emphasize the specificity of ethanol's actions in the CNS, and the fact that adaptation to ethanol can occur at sites that are initially affected by ethanol.

Interaction of agonists with some receptors in the CNS does not modulate AC activity, but results in changes in calcium mobilization. The first step in this action appears to be breakdown of membrane-localized polyphosphoinositide (PI) to yield inositol phosphates, which then mediate calcium mobilization. Ethanol in vitro selectively decreased the potency of muscarinic cholinergic agonists to stimulate PI breakdown in cortex and hippocampus. In animals physically dependent on ethanol, the number of cortical muscarinic cholinergic receptors was increased, and as a result, the efficacy of carbachol to stimulate PI turnover in cerebral cortex was increased. This adaptive response was the first demonstration of a functional correlate to a change in receptor number in ethanol-treated animals, and suggested a mechanism for increased sensitivity to acetycholine which may underly particular symptoms of ethanol withdrawal.

2. Ethanol Effects on Membrane-Bound Enzymes

Ethanol and other alcohols, in vitro, were found to inhibit (Na^+/K^+) ATPase activity, and the relationship of inhibitory potency to alcohol chain length and lipid solubility suggested that alcohol inhibition may involve a direct interaction with the protein, rather than solely an interaction with membrane lipids. Although others have suggested that catecholamines potentiate the actions of ethanol, no evidence was found to suggest that low, physiologically attainable concentrations of ethanol significantly inhibit ATPase activity either in the presence or absence of catecholamines, and the role of this enzyme activity per se in the CNS actions of ethanol remains open to question. However, chronic ethanol treatment selectively altered the sensitivity of the neuronal form of (Na^+/K^+)ATPase to inhibition by ouabain. Ouabain inhibition depends on membrane lipid properties, and the changes in this characteristic of (Na^+/K^+)ATPase may serve as a reliable probe for alterations in lipid properties, within specific areas of the neuronal membrane, which are induced by chronic ethanol ingestion. Such changes may be associated with particular aspects of ethanol tolerance and/or dependence.

Ethanol was also found to selectively inhibit one form of monoamine oxidase (MAO-B) in human tissues, apparently by an action on the lipid microenvironment of this form of the enzyme. This enzyme activity was inhibited substantially by low (50 mM) concentrations of ethanol, and resultant changes in turnover of brain amines may contribute to certain of ethanol's effects. The studies of ethanol effects on enzyme activities also

provided basic information regarding the properties of the various forms of (Na^+/K^+)ATPase and MAO (A and B).

In addition, studies focusing on basic mechanisms of regulation of neuronal activity were performed. It was shown that glial transport of the excitatory amino acid neurotransmitter, glutamate, is altered by disruption of glial glutamate metabolism. Such studies define the basic mechanisms of CNS function and will allow further understanding of the sites of action of ethanol in the CNS.

3. Ethanol Effects on the GABA-BDZ-Barbiturate Receptor/Chloride Ionophore

Ethanol inhibits the binding of ^{35}S-t-butylbicyclophosphorothionate (^{35}S-TBPS) to membranes from cerebral cortex and cerebellum of C57Bl mice. This ligand is believed to bind to a regulatory site associated with the GABA receptor-coupled chloride ion channel, and changes in binding of TBPS are linked to changes in the function of that channel. Binding of this ligand was quite sensitive to ethanol, in that concentrations of ethanol attainable in vivo (50-100 mM) produced substantial inhibition. GABA and pentobarbital also inhibited TBPS binding, and their effects (IC_{50}) were not altered by ethanol (100 mM). Nevertheless, the absolute decrease in TBPS binding was enhanced in the presence of ethanol plus GABA or pentobarbital, compared to that seen with GABA or pentobarbital alone, and this event may be associated with potentiation of the behavioral effects of GABA or barbiturates by ethanol. Chronic in vivo treatment of animals with ethanol did not alter TBPS binding, or the response of this binding to ethanol, GABA, or barbiturates.

In contrast, although benzodiazepine (^{3}H-flunitrazepam) (Flu) binding to cortical and cerebellar membranes was not altered by in vivo chronic ethanol treatment of mice, the ability of pentobarbital to stimulate ^{3}H-Flu binding was decreased in these animals. Chronic in vivo pentobarbital treatment had a similar effect, and also decreased ^{3}H-Flu binding per se. These studies suggest that certain ethanol's CNS pharmacological effects may be mediated by an interaction with the chloride ionophore linked to the GABA-benzodiazepine-barbiturate receptor complex. In animals that are tolerant to and dependent on ethanol, the function of this complex is altered, but the change, which may represent an adaptation to the initial effect of ethanol, seems to occur at a site different from the site of action of ethanol. These studies suggest a biochemical basis for certain signs of ethanol withdrawal, as well as for the development of tolerance and cross-tolerance between ethanol and barbiturates. A clearer understanding of the mechanism by which ethanol interacts with this receptor complex may allow for development of more rational therapies for treatment of alcohol withdrawal symptomatology.

4. Neurohypophyseal Peptides and Ethanol Tolerance

Our previous work had shown that exogenously administered arginine vasopressin (AVP) can maintain tolerance to ethanol in animals, once that tolerance has been established, in the absence of further intake of ethanol. In those studies, the peptides were administered to mice systemically. Although AVP analogs with reduced peripheral effects could maintain tolerance, a more direct evaluation of the site of action of AVP--i.e., central vs peripheral--was undertaken. It was found that intracerebroventricular (icv) injection of

AVP, at a dose that had no detectable systemic effects, maintained ethanol tolerance. Injection of animals (mice or rats) with a specific peptide antagonist (also icv) resulted in an enhanced rate of loss of tolerance, thus implicating endogenous hormone in the maintenance of tolerance. AVP was also found to maintain a form of ethanol tolerance that has been called "environment-dependent." This form of tolerance consists of a conditioned compensatory response to the effect of ethanol, and is thought to have a prominent "learned" component. The finding that AVP maintains this form of tolerance, as well as "environment- independent" tolerance (see Tabakoff, Melchior and Hoffman, Alc: Clin. Exptl. Res. 6:252, 1982 for further description of various forms of tolerance), suggests certain common mechanisms for the two forms of tolerance and supports the hypothesis that tolerance and memory or learning, as CNS adaptive processes, also share certain underlying mechanisms. Interestingly, AVP inhibited the rate of acquisition of environment-dependent tolerance, possibly by interfering with the animals' perception of the cues associated with ethanol administration. We have initiated studies of the biochemical effect of vasopressin in the CNS, for eventual comparison (e.g., by structure-activity analysis) to the effect of the hormone and its analogs on ethanol tolerance, These studies may lead to the design of therapies to manipulate the development or dissipation of ethanol tolerance and, as a consequence, to influence ethanol intake.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00700-01 LSN

PERIOD COVERED
November 1, 1984 to September 30, 1985

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	LSN, NIAAA
	B. Tabakoff	Acting Chief	LSN, NIAAA

COOPERATING UNITS *(if any)*
University of Illinois Rockford Medical School (B. Salafsky, J.D. Rothstein); Westside VA, Chicago (F. DeLeon-Jones).

LAB/BRANCH
Laboratory for Studies of Neuroadaptive Processes

SECTION
Section of Neurobiology

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

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.)*

Ethanol perturbs the structure of neuronal membrane lipids, and its CNS effects may arise secondarily as a result of changes in the function of membrane-bound enzymes. At high concentrations ethanol inhibits brain (Na-+, K-+) ATPase activity, and we found that inhibition of mouse synaptosomal ATPase activity by alcohols does not depend solely on interactions with membrane lipids; ethanol may also interact directly with the protein. Our studies showed no potentiation by norepinephrine of ethanol-induced inhibition of ATPase in mouse or rat brain. Since substantial inhibition of activity occurs only at high ethanol concentrations, the role of enzyme inhibition per se in the CNS actions of ethanol remains open to question. However, other aspects of ATPase activity can serve as a probe of changes in membrane structure, following ethanol exposure, which may be associated with tolerance or physical dependence.

ATPase exists in two forms in the brain, with high and low affinity for the inhibitor, ouabain. The high-affinity component is believed to be localized in neuronal membranes. Following chronic ethanol treatment, there was a selective increase in affinity for ouabain of this component, most likely resulting from changes in the lipid microdomain of this form of the enzyme. Another membrane-bound enzyme, monoamine oxidase (MAO), also exists in two forms, A and B. Ethanol selectively inhibited the B form of the enzyme in human brain and platelet tissue by perturbation of membrane lipids, supporting the hypothesis that the two forms of MAO are distinguished by their lipid environments. Ethanol at 50 mM significantly inhibited enzyme activity, and inhibition of MAO-B could play a role in mediating the CNS effects of ethanol. This project also included studies of glial regulation of neurotransmitter metabolism, adding to our understanding of basic mechanisms of regulation of neuronal activity.

PROJECT DESCRIPTION:

Investigators:

P. Hoffman	Section Chief	LSN, NIAAA
B. Tabakoff	Acting Chief	LSN, NIAAA
P. Nhamburo	Guest Researcher	LSN, NIAAA
B. Salafsky	Professor	Univ. of Illinois Rockford Med. Sch.
F. DeLeon-Jones	ACOS for Research	Westside VA Medical Center, Chicago
J. Rothstein	Medical Resident	Univ. of Illinois Rockford Med. Sch.

Objectives:

Ethanol can perturb the structure of neuronal membrane lipids and, secondarily, alter the activity of membrane-bound enzymes. If the effect of ethanol on enzyme activity is lipid-mediated, then the activities of these enzymes can serve as probes to elucidate the site of action of ethanol within the membrane, or the changes in membrane properties that occur as adaptive responses to the chronic presence of ethanol. Alternatively, ethanol could directly affect the characteristics of enzyme proteins. In order to correctly interpret ethanol-induced alterations in enzyme activity, it is necessary to understand the mechanism of action of ethanol. Furthermore, in many instances, high in vitro concentrations of ethanol are necessary to alter the activity of membrane-bound enzymes. Thus, ethanol, at concentrations attainable in vivo, may have little effect on enzyme activity, and the question arises as to whether changes in enzyme activity per se are actually involved in the CNS effects of ethanol, or whether the activities of such enzymes are most useful as reflections of ethanol-induced changes in membrane lipid properties (e.g., see Levental and Tabakoff: J. Pharmacol. Exp. Ther. 212: 315-319, 1980).

In this project the effects of ethanol on membrane-bound enzymes in the CNS and periphery are examined. The enzymes chosen are (Na^+, K^+)ATPase and monoamine oxidase (MAO). The studies are designed to elucidate the mechanism by which ethanol alters the activity of these enzymes, and the possible physiological importance of ethanol-induced changes in enzyme activity. In addition to investigations of enzymes involved in neuronal function and synaptic transmission, studies of basic mechanisms of regulation of neurotransmitter metabolism were undertaken.

Methods Employed:

(Na^+,K^+)ATPase activity. Male C57Bl mice (22-25 g) and male Wistar rats (250-300 g) were used in these experiments. Animals were decapitated, and their brains dissected in the cold. A synaptosomal preparation of whole brain (minus cerebellum and spinal cord) was obtained by the method of Cotman (Methods Enzymol. 31:445-452, 1974), using sucrose-Ficoll density gradient centrifugation. Synaptosomal pellets were frozen, thawed, and subjected to hypoosmotic shock to provide a synaptosomal membrane preparation. For studies of norepinephrine (NE) effects, cerebral cortical tissue from mice or rats was homogenized in medium containing 0.1% deoxycholate, and a crude synaptosomal membrane pellet was prepared.

(Na^+,K^+)ATPase activity was determined as previously described (Levental and Tabakoff: J. Pharmacol. Exp. Ther. 212:315-319, 1980), in the presence and absence of 100 mM NaCl and 5 mM KCl. Inorganic phosphate was extracted and quantitated by described methods (Penniall: Anal. Biochem. 14:87-90, 1966). (Na^+, K^+)ATPase activity was calculated as the difference between the quantity of inorganic phosphate liberated in the presence and absence of Na^+ and K^+.

MAO activity. MAO activity was measured in human platelets and in human brain tissue obtained at autopsy. Platelets were prepared from whole blood (50 ml) obtained by venipuncture from healthy volunteers who had given informed consent. Human brain tissue (frontal cortex) was kindly provided by Drs. Peter Valverius and Stefan Borg, Karolinska Institute, Stockholm, Sweden. Brain tissue was obtained within 4 hours of death and was stored at -70° C until used.

MAO activity in preparations of both tissues was assayed by a modification of the spectrophotometric method of Tabakoff and Alivisatos (Anal. Chem. 44:427-428, 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. 12:1439-1441, 1963).

Regulation of glutamate transport. Glutamate transport into striatal tissue was studied following inhibition of glutamine synthetase with methionine sulfoximine (MSO). Male Sprague-Dawley rats were injected intraventricularly with MSO and were killed at various times after the injections. Brains were removed and striatal tissue was sliced using a McIlwain tissue chopper. In some instances synaptosomal, glial, or neuronal fractions were specifically separated by techniques of differential centrifugation and cell isolation. To assess glutamate uptake, tissue was incubated with ^{14}C-glutamate and radioactivity was assessed after filtration or centrifugation.

Major Findings:

Ethanol and other short-chain aliphatic alcohols inhibited synaptosomal (Na^+, K^+)ATPase activity, and inhibition increased with increasing alcohol chain length. Such results are consistent with the hypothesis that partitioning of the alcohols into the neuronal membrane is important for inhibition of ATPase activity. However, more detailed analysis of the data revealed a relationship between inhibitory potency and alcohol chain length or membrane:water partition coefficient that did not support an interpretation that inhibition of enzyme activity depends solely on the interaction of alcohols with membrane lipids. Thus, a direct interaction of ethanol with the enzyme protein may be involved in inhibition of ATPase activity. This hypothesis was supported by our finding that ethanol also inhibited Li-dependent phosphatase activity. Measurement of this activity circumvents the conformational interconversions necessary for ATPase activity, and that would presumably be influenced by the physical state of the membrane lipids surrounding the enzyme.

In these experiments, relatively high concentrations of ethanol (e.g., 250 mM) were necessary to produce substantial inhibition of ATPase activity, as has previously been reported. It has also been reported, however, that catecholamines, especially norepinephrine (NE), potentiate ethanol-induced inhibition of ATPase from rat brain. Thus, in noradrenergically innervated brain areas, low

(e.g., 50 mM) concentrations of ethanol could significantly inhibit enzyme activity, and this inhibition could contribute to the CNS effects of ethanol in vivo. However, in our study, NE did not alter ethanol inhibition of ATPase in rat or mouse synaptosomal membranes. Therefore, the importance of changes in ATPase activity in the in vivo effects of ethanol remains open to question.

In addition to inhibiting ATPase activity, ethanol also alters other characteristics of the enzyme, some of which appear to be associated with its lipid microenvironment. ATPase in brain has recently been found to exist in two forms, with high and low affinity for the inhibitor, ouabain. We found that ethanol, in vitro, increased the affinity of the high-affinity form of the enzyme for ouabain; after chronic ethanol feeding of mice, a much larger (about eightfold) increase in affinity for ouabain of the high-affinity form of the enzyme was observed. This was a selective change in the properties of the high-affinity form of ATPase, which is thought to be localized neuronally. Since ouabain affinity is regulated by lipids, these results may be interpreted to indicate that chronic ethanol treatment selectively alters the lipid environment of the neuronal ATPase. This change provides a clear-cut means to probe a site within the neuronal membrane at which adaptive responses to ethanol occur. The results also suggest that the two forms of ATPase may differ primarily with respect to their membrane microenvironments.

Another enzyme inhibited by ethanol is monoamine oxidase (MAO). MAO also exists in two forms with differing substrate specificities, known as MAO-A and MAO-B. Platelet MAO has the characteristics of MAO-B while brain contains both forms of the enzyme. The nature of the differences between MAO-A and B is not clear, but some evidence indicates that the membrane environments of these two forms of the enzyme differ. Ethanol was found to selectively inhibit the B-form of MAO in human platelet and brain tissue. The inhibitory potencies of ethanol and other alcohols suggested that inhibition was related to the membrane-perturbing properties of the alcohols, supporting the hypothesis of differing lipid microenvironments for the two forms of the enzyme. In contrast to the ATPase, it was found that, at substrate concentrations approximating endogenous levels, low concentrations of ethanol (e.g., 50 mM) could substantially inhibit MAO activity. One substrate tested was phenylethylamine (PEA), a trace amine believed to be important for the reinforcing properties of various drugs. Inhibition by ethanol of PEA deamination in the CNS could therefore contribute to certain of ethanol's behavioral and physiological effects.

The studies of glutamate uptake revealed that glutamine synthetase (GS), an enzyme localized in glial cells, plays an important role in regulating glutamate uptake into glial cells. When GS was inhibited, glutamate uptake into glial (but not neuronal) cells was increased. It appears that when glutamate metabolism via the glutamate-glutamine cycle is altered (i.e., by inhibition of GS), changes in glial glutamate uptake occur as an adaptive response. These results enhance the understanding of basic mechanisms of neurotransmitter regulation in the CNS.

Significance to Biomedical Research and the Program of the Institute:

The findings resulting from this project indicate that, while inhibition of (Na^+, K^+)ATPase per se is not clearly implicated in the in vivo physiological effects of ethanol, inhibition of another membrane-bound enzyme (MAO-B) may be

important for certain of these effects. On the other hand, the selective alterations in the sensitivity to ouabain of neuronal (Na^+, K^+)ATPase indicate that chronic ethanol treatment results in specifically localized alterations in neuronal membrane properties, which can be probed by the use of enzyme activity, and which may be important in particular aspects of adaptation to the chronic effects of ethanol.

These studies support the hypothesis that different "forms" of membrane-bound enzymes reflect differences in their membrane lipid microenvironments and thus provide basic information about enzyme properties. The studies of glutamate metabolism also add to a basic understanding of the factors that regulate neurotransmitter function. Glutamine, as a putative excitatory neurotransmitter in the CNS, plays an important role in modulation of neuronal activity, and an understanding of the basic mechanisms of its regulation, as well as that of other neurotransmitters, is crucial to further evaluation of the effects of ethanol in the CNS.

Proposed Course:

The changes in sensitivity of (Na^+, K^+)ATPase to ouabain after chronic ethanol treatment will be characterized in detail, including studies of ouabain binding. These alterations are planned to be used for characterization of changes in neuronal membrane lipid properties associated with ethanol tolerance. For example, we plan to exchange lipids between synaptosomal preparations of control and ethanol-tolerant animals, and to measure ouabain inhibition of (Na^+, K^+)ATPase and ouabain binding in these tissues.

The role of MAO-B inhibition in the physiological and/or behavioral effects of ethanol will be analyzed by measuring levels and turnover of PEA and other amines in brains of animals following various acute doses of ethanol in vivo.

Studies of the regulation of glutamate and aspartate metabolism by glial mechanisms have been completed.

Publications:

Rothstein, J.D., and Tabakoff, B.: Glial and neuronal glutamate transport following glutamine synthetase inhibition. Biochem. Pharmacol. 34:73-79, 1985.

Tabakoff, B., Lee, J.M., DeLeon-Jones, F., and Hoffman, P.L.: Ethanol inhibits MAO-B activity in human platelet and brain tissue. Psychopharmacology (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00701-01 LSN

PERIOD COVERED
November 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Ethanol Actions at the GABA-BDZ-Barbiturate Receptor/Chloride Ionophore

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

PI:	P. Hoffman	Section Chief	LSN, NIAAA
	B. Tabakoff	Acting Chief	LSN, NIAAA
Other:	S. Culp	Research Chemist	LSN, NIAAA

COOPERATING UNITS *(if any)*
University of Illinois Rockford Medical School (B. Salafsky).

LAB/BRANCH
Laboratory for Studies of Neuroadaptive Processes

SECTION
Section of Neurobiology

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

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	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.)*

Many pharmacological effects of ethanol have been postulated to be mediated via GABA-containing neuronal systems in the CNS. The GABA receptor exists as part of a complex containing receptors for benzodiazepines (BDZ), barbiturates, and the chloride ion channel. To examine the effects of ethanol on this complex, we have studied the binding properties of 35-S-t-butylbicyclophosphorothionate (35-S-TBPS), a ligand that interacts with the regulatory site of the chloride ion channel in membranes of cortex and cerebellum of C57Bl mice in the presence and absence of ethanol. In vitro addition of ethanol produced a dose-dependent inhibition of TBPS binding in both cerebellum and cortex (IC-50 approximately 300 mM). At a concentration of ethanol that is physiologically attainable, i.e., 100 mM, there was substantial inhibition of TBPS binding. GABA and pentobarbital also inhibited the binding of 35-S-TBPS in cortex and cerebellum, and ethanol did not potentiate the actions of these agents. Our initial results indicate that after chronic in vivo ethanol administration, which produces functional tolerance and physical dependence, tolerance to the in vitro effects of ethanol on TBPS binding does not occur, and the effects of GABA and barbiturates are also unchanged. In contrast to the results with TBPS, chronic ethanol treatment of mice resulted in a reduced ability of pentobarbital, added to assays in vitro, to stimulate BDZ (3-H-Flunitrazepam) (Flu) binding in cortex and cerebellum. Similar results were found in animals that were chronically fed barbiturates. The results support the hypothesis that certain pharmacological effects of ethanol may result from interactions with the GABA-BDZ-barbiturate receptor complex. Chronic treatment with ethanol results in possible adaptive changes that occur at a site other than that originally influenced by ethanol, but that alter the function of the complex as a whole. These biochemical alterations may be associated with various aspects of tolerance, cross-tolerance, and physical dependence on ethanol.

PROJECT DESCRIPTION:

Investigators:

B. Tabakoff	Acting Chief	LSN, NIAAA
P. Hoffman	Section Chief	LSN, NIAAA
S. Liljequist	Guest Researcher	LSN, NIAAA
S. Culp	Research Chemist	LSN, NIAAA
B. Salafsky	Professor	Univ. of Illinois Rockford Med. Sch

Objectives:

Many of the behavioral and electrophysiological effects of ethanol have bee postulated to be mediated by neuronal systems utilizing the neurotransmitte gamma-aminobutyric acid (GABA). The receptor for GABA is part of a complex tha also contains receptors for barbiturates and benzodiazepines (BDZ), as well as th chloride ion channel. Benzodiazepines are widely used to treat ethanol withdrawa symptoms, supporting a role for this receptor complex in the responses of the CN to chronic ethanol exposure. The purpose of this project is to investigate th direct effects of ethanol on the GABA-benzodiazepine-barbiturate receptor comple and the associated chloride ionophore in the brain.

Methods Employed:

Chronic ethanol administration. Ethanol was administered chronically to mal C57Bl mice according to a previously described protocol (Ritzmann and Tabakoff J. Pharmacol. Exp. Ther. 199:158-170, 1976). An ethanol-containing liquid die was fed to mice for 7 days. Animals were either sacrificed on the morning of th eighth day, while still intoxicated, or were sacrificed 24 hours after the ethano diet was substituted by a diet containing an equicaloric amount of sucrose. Dur ing this 24-hour period, the animals were observed and the characteristic symptom of ethanol withdrawal were quantitated. Control mice were pair-fed a liquid die containing sucrose in amounts equicaloric to the ethanol.

Chronic barbiturate administration. Male C57Bl mice were housed individually an fed milled laboratory chow. Phenobarbital was added to the chow fed to the ex perimental group. Animals were sacrificed on the morning of the seventh day o treatment.

Receptor binding experiments. After decapitation of the mice, brains were quickl removed, and the cerebral cortex and cerebellum were dissected on ice. Brai membranes were prepared as described by Supavilai and Karobath (J. Neurosci 4:1193-1200, 1984) for experiments using [^{35}S]-t-butylbicyclophosphorothionat ([^{35}S]-TBPS). For studies of BDZ binding ([^{3}H]-Flunitrazepam)([^{3}H]-Flu) brain tissue was homogenized in 20 volumes of 0.32 M sucrose and HEPES buffer After a low-speed centrifugation, the supernatant was recentrifuged at 48,000 x and the pellet was resuspended in distilled water. The suspension was recentri fuged at 48,000 xg and the pellet washed five times with phosphate buffer.

The binding of [^{35}S]-TBPS was performed as described by Squires et al. (Squires Casida, Richardson, and Saederup: Mol. Pharmacol. 23:326-336, 1983) and Supavila

and Karobath (1984). Brain membranes were suspended in Tris-citrate buffer (pH 7.4) containing 100 mM KBr. Incubations in the presence of various concentrations of ligand were carried out at 21° C for 90 minutes. Duplicate aliquots of the incubation mixture were filtered over Whatman GF/B filters. The filters were washed with ice-cold buffer, placed in scintillation vials, and dried overnight. Radioactivity was quantitated by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 μM picrotoxin and represented approximately 15% of total binding. Binding of [^{3}H]-Flu was measured by incubating a membrane suspension at 0° C for 75 minutes with varying concentrations of [^{3}H]-Flu. Nonspecific binding was determined with 10 μM diazepam. Filtration and quantitation of binding were performed as described above. In some instances, pentobarbital was added to the assays, and in others, Type 1 and Type 2 BDZ receptors were distinguished by measuring displacement of [^{3}H]-Flu binding by CL-218,872.

Major Findings:

The ligand [^{35}S]-TBPS is believed to bind to a membrane site closely associated with the chloride ionophore of the GABA-BDZ receptor system. Modulation of the binding of this ligand indicates altered function of the chloride ion channel. Addition of ethanol in vitro to binding assays resulted in a dose-dependent inhibition of [^{35}S]-TBPS binding to cerebellar and cortical membranes of C57Bl mice. The IC_{50} value for ethanol inhibition of binding in each brain area was about 300 mM. At ethanol concentrations that can be attained in vivo, i.e., 50 to 100 mM, there was a substantial decrease in [^{35}S]-TBPS binding. Both GABA and pentobarbital also inhibit [^{35}S]-TBPS binding. In order to assess whether the TBPS binding site may be involved in the previously described potentiation by ethanol of the behavioral effects of GABA and pentobarbital, inhibition of TBPS binding by GABA and pentobarbital was also studied in the presence of 100 mM ethanol. Although ethanol per se inhibited TBPS binding, it did not alter the efficacy of either GABA or the barbiturate to inhibit TBPS binding. However, the absolute amount of TBPS binding in the presence of a given concentration of GABA or pentobarbital was further reduced upon addition of ethanol, and thus the chloride channel, in addition to being a site of action of ethanol itself, may represent an important site at which ethanol can influence the behavioral effects of the other agents.

Animals given ethanol in a liquid diet for 7 days were tolerant to and physically dependent on ethanol. In these animals, [^{35}S]-TBPS binding in cortex and cerebellum was unchanged, and the in vitro effects of ethanol, GABA, and pentobarbital on TBPS binding were also not altered. Similar results were obtained in animals sacrificed at the time of withdrawal, while they were still intoxicated, or in animals sacrificed 24 hours after withdrawal, when overt withdrawal signs had peaked and dissipated. Thus, changes in the properties of the recognition site for TBPS do not appear to be associated with functional tolerance to or physical dependence on ethanol. In this instance, no apparent adaptation occurred at the initial site of action of ethanol in the CNS.

On the other hand, chronic ethanol treatment of animals in vivo did alter the properties of other components of the GABA-BDZ receptor complex. Although, in tissue obtained from animals 24 hours after withdrawal, there was no change in [^{3}H]-Flu binding to the BDZ receptor in cortex or cerebellum, there was a

decreased stimulation of [^{3}H]-Flu binding by pentobarbital, added in vitro, i the ethanol-withdrawn mice. Interestingly, chronic phenobarbital treatment o animals also resulted in a reduced ability of pentobarbital to stimulate [^{3}H] Flu binding. This latter treatment also produced a decrease in total number o BDZ binding sites by decreasing the number of Type 1 sites in cerebellum and th number of Type 2 sites in cortex.

Significance to Biomedical Research and the Program of the Institute:

Our results support the hypothesis that the GABA-BDZ receptor system in the brai may be involved in some of ethanol's pharmacological actions. This observation i important since evidence is increasing that disturbances in central GABAergi neurotransmission may be involved in the epileptiform seizure activity frequentl observed during alcohol abstinence in chronic alcoholics. The results als provide insight into the mechanisms of CNS adaptation to the chronic presence o ethanol. Although ethanol, like barbiturates, can directly affect the binding o a ligand to the regulatory site for the chloride ion channel, indicating altera tion in the function of the whole complex, adaptation to this effect appears t occur at another site, i.e., there is a decreased ability for barbiturates t modify BDZ binding. This change may also result in altered function of the com plex as a whole. Overall, these changes in the GABA-BDZ-barbiturate recepto complex could well contribute to certain ethanol withdrawal signs. These result suggest possible mechanisms for the observed cross-tolerance that occurs amon ethanol, BDZ's, and barbiturates. Understanding the basis of these interaction may allow the development of more rational therapies for treatment of alcoho withdrawal symptoms.

Proposed Course:

We will investigate the effects of acute (in vitro) and chronic ethanol exposur on the binding properties of other ligands known to interact with recognitio sites within the GABA-BDZ receptor system. Since it has been shown that thes interactions are highly dependent on experimental conditions used, several proce dures of membrane preparation and various assay conditions will be tested. Fur thermore, we plan to evaluate the effects of ethanol on the functional conse quences of activation of the GABA-BDZ receptor complex, such as chloride flux.

Publications:

Liljequist, S., and Tabakoff, B.: Binding characteristics of ^{3}H-Flunitrazepa and CL218,872 in cerebellum and cortex of C57Bl mice made tolerant to and depend ent on phenobarbital or ethanol. Alcohol 2:215-220, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AA 00702-01 LSN

PERIOD COVERED
November 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*
Ethanol Modification of Neurotransmitter Receptor-Effector Coupling Processes

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

PI:	P. Hoffman	Section Chief	LSN, NIAAA
	B. Tabakoff	Acting Chief	LSN, NIAAA
Others:	C. Chung	Staff Fellow	LSN, NIAAA
	E. Majchrowicz	Research Chemist	LPS, NIAAA
	H. Moss	Medical Staff Fellow	LCS, NIAAA
	P. Martin	Visiting Scientist	LCS, NIAAA

COOPERATING UNITS *(if any)*
NIMH, CPB (L. Tamarkin); University of Illinois Med. Ctr. (J.M. Lee); Sapporo Med. College (T. Saito).

LAB/BRANCH
Laboratory for Studies of Neuroadaptive Processes

SECTION
Neurobiology

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

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.)*

The effects of ethanol on several neurotransmitter receptor-effector coupling systems were investigated in order to elucidate the specificity of ethanol's effects, the site(s) and mechanisms of ethanol action, and the possible role of these systems in adaptation to the chronic effects of ethanol (i.e., tolerance and/or physical dependence). In cerebral cortex of C57Bl mice, ethanol increased the activity of beta-adrenergic receptor-coupled adenylate cyclase (AC) in a dose-dependent manner. Specific sites of ethanol action were: the beta-adrenergic receptor N(s) (the guanine nucleotide-binding protein) and the catalytic unit of AC. Ethanol also promoted the interaction of guanine nucleotide-loaded N(s) with AC. Preliminary results showed that in ethanol-tolerant and -dependent mice, as well as in rats, stimulation of cortical AC by guanine nucleotides and isoproterenol (ISO) was reduced, suggesting an adaptive response at the biochemical level. Similar results (i.e., reduced sensitivity to stimulation by guanine nucleotides and ISO) were found for rat pineal gland AC, indicating a generalized effect on this receptor-effector system. In contrast, ethanol, acutely or chronically, did not alter opiate inhibition of AC in mouse striatum. The effect of ethanol seems to be relatively specific for systems using N(s), as opposed to N(i). Ethanol also affected receptor coupling to another biochemical transducer, phosphatidylinositol (PI) turnover. Ethanol inhibited this activity and, after chronic in vivo treatment of mice with ethanol, there was increased sensitivity of PI turnover to stimulation by cholinergic agonist, but not by a noradrenergic agonist. These changes paralleled previously observed alterations in ligand binding, demonstrating a functional correlate of ethanol-induced alterations in receptor number in the CNS. This change may be associated with specific symptoms of ethanol withdrawal. Overall, the studies illustrate that ethanol has specific sites of action in the CNS, and that adaptation occurs at these sites after chronic treatment of animals with ethanol, which results in tolerance and physical dependence.

PROJECT DESCRIPTION:

Investigators:

B. Tabakoff	Acting Chief	LSN, NIAAA
P. Hoffman	Section Chief	LSN, NIAAA
C. Chung	Staff Fellow	LSN, NIAAA
F. Moses	Guest Researcher	LSN, NIAAA
E. Majchrowicz	Research Chemist	LPS, NIAAA
H. Moss	Medical Staff Fellow	LCS, NIAAA
L. Tamarkin	Research Biologist	CPB, NIMH
J. Lee	Medical Student	Univ. of Illinois Med. Center
T. Saito	Asst. Professor	Sapporo Med. Coll., Sapporo, Japan
P. Martin	Visiting Scientist	LCS, NIAAA

Objectives:

Ethanol has been postulated to produce its CNS effects (e.g., intoxication) by modifying the properties of neuronal membrane lipids (i.e., increasing fluidity). When animals were chronically fed ethanol such that they were tolerant to and physically dependent on ethanol, their brain membranes were resistant to the fluidizing effects of ethanol, suggesting a biophysical basis for adaptation to ethanol (Chin and Goldstein: Science 196:684, 1972). However, while lipids form the structural matrix of neuronal membranes, the functional moieties of these membranes are proteins, such as receptors and enzymes. The activities of these membrane-bound proteins, many of which play crucial roles in synaptic transmission, are dependent on their membrane lipid environments. Thus, ethanol may modify the activity of the proteins indirectly, via effects on lipid properties, or by direct actions on the proteins per se. One complex of membrane-bound proteins that mediates the actions of many hormones and neurotransmitters is the receptor-coupled adenylate cyclase (AC) system. Activation of receptors and stimulation or inhibition of AC activity involve several protein-protein interactions that have been intensively investigated. Within the framework of the model developed to describe these interactions, the effects of ethanol can be defined, and we had previously demonstrated particular sites of action for ethanol in one receptor-coupled AC system in the brain (Luthin and Tabakoff: J. Pharmacol. Exp. Ther. 228:579-587, 1984.

We have several objectives in this project. We wish to determine the specificity of the actions of ethanol on receptor-coupled AC systems: whether ethanol affects all receptor-AC systems (i.e., stimulatory vs. inhibitory) in the same way, and by similar mechanism(s). In those receptor-coupled systems in which ethanol modifies activity, we wish to determine the sites and mechanism(s) of action of ethanol (e.g., receptor; guanine-nucleotide-binding proteins, N_s,N_i; catalytic unit of AC). We also plan to determine the effect of ethanol in systems in which receptors are coupled to effectors other than AC. Specifically, the effect of ethanol on stimulation of phosphatidylinositol turnover by cholinergic and noradrenergic agonists is of interest. These studies will provide further insight into the specificity and mechanism of ethanol's actions in the CNS. The

receptor-effector coupling systems will also be investigated after chronic in vivo treatment of animals with ethanol, leading to the development of functional tolerance and physical dependence. Such studies will determine whether 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 Employed:

For chronic ethanol treatment, male C57Bl mice (23 to 25 g) were individually housed and fed a liquid diet containing ethanol or an equicaloric amount of sucrose (controls) for several days (Ritzmann and Tabakoff: J. Pharmacol. Exp. Ther. 199:158-170, 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, for studies of opiate effects on AC, at 24 hours after withdrawal. In this case, withdrawal symptoms were monitored in the ethanol-treated mice. Male Sprague-Dawley rats were treated chronically with ethanol by gavage, and following cessation of ethanol treatment, withdrawal symptoms were monitored as previously described (Majchrowicz, E.: Psychopharmacology 43: 245-254, 1975). Animals were sacrificed at the time when withdrawal symptoms peaked.

Adenylate cyclase assay. Animals were killed by decapitation, brains were removed, and a 48,000 xg membrane fraction was prepared as previously described (Luthin and Tabakoff: J. Pharmacol. Exp. Ther. 228:579-587, 1984). Adenylate cyclase activity was measured according to methods described in our laboratories, by determining the production of 32-P-cyclic AMP from 32-P-ATP (Tabakoff and Hoffman: J. Pharmacol. Exp. Ther. 208:216-222, 1979).

Phosphatidylinositol turnover (polyphosphinositide breakdown). Phosphatidylinositol breakdown was measured according to a modification of a previously described method (Janowsky, Labarca, and Paul: Life Sci. 35:1953-1961, 1984). Briefly, animals were decapitated, brains quickly removed, and the appropriate areas (cortex, hippocampus, striatum) dissected on ice. Slices (350 μm x 350 μm) were prepared using a McIlwain tissue chopper. The slices were incubated for 30 minutes at 37° C in Krebs-Henseleit buffer and were then incubated in fresh buffer containing 0.3 μM ^{3}H-myoinositol for 1 hour at 37° C. Slices were washed, and 20-30 μl of packed slices were transferred to assay tubes containing 10 mM LiCl and various concentrations of neurotransmitter or other agents. The reaction was carried out for 1 hour at 37° C and terminated by addition of a solution of $CHCl_3$/MeOH (1:2). Following mixing, water and $CHCl_3$ were added to each tube, and tubes were centrifuged to separate the aqueous and organic layers. The labeled inositol phosphates were isolated from the aqueous layer by column chromatography.

Major Findings:

Ethanol increased basal, guanine nucleotide-stimulated, and isoproterenol-stimulated AC activity in cerebral cortex, and the action of ethanol was enhanced in the presence of guanine nucleotides. Ethanol had several sites of action in this system, similar to what had been reported for the striatal dopamine-sensitive

AC system (Luthin and Tabakoff: J. Pharmacol. Exp. Ther. 228:579-587, 1984). First, ethanol enhanced the rate of activation of AC by guanine nucleotides, suggesting that ethanol may promote the dissociation of cortical N_s into its alpha and beta (gamma) subunits. Second, ethanol had a direct stimulatory effect on detergent-solubilized cortical AC, i.e., on the catalytic unit of the enzyme per se. Third, ethanol altered the equilibrium for the interaction of guanine nucleotide-activated N_s with the catalytic unit of AC, to favor formation of the active N_sAC complex. Finally, in cerebral cortex, ethanol appeared to alter the interaction of isoproterenol (ISO) with the beta-adrenergic receptor. That is, ethanol caused a shift to the right of the dose-response curve for ISO stimulation of cortical AC activity. Preliminary results suggest that ethanol may affect agonist binding to beta-adrenergic receptors via an interaction with N_s, rather than a direct effect on the receptor, and investigation of this interaction is continuing. Overall, these results indicated that, acutely, ethanol altered the cortical beta-adrenergic receptor-AC system by acting at specific sites, which were in part similar to the sites of action in striatum.

We have also begun to evaluate changes in the cortical beta-adrenergic receptor-AC system in animals treated chronically with ethanol. Our initial results are consistent with a defect in the function of N_s in these animals. For example, there was a decreased response of cortical AC to stimulation by guanine nucleotides and by isoproterenol in animals sacrificed at the time of ethanol withdrawal. Stimulation of cortical AC by ethanol was also reduced in tissues from these animals, as compared to controls. The studies described above were performed using mice that were given ethanol in a liquid diet. More recently we have initiated studies in another species (rats), in which ethanol was administered chronically by gavage. These animals were sacrificed at the time of peak withdrawal symptomatology and cortical AC activity was assessed. Similar to what was observed in the mice, the stimulation of AC by guanine nucleotides was reduced in tissue obtained from animals treated chronically with ethanol, and the dose-response curve for isoproterenol was shifted to the right. To further assess the generality of ethanol-induced alterations in the coupling of beta-adrenergic receptors to AC, we have initiated studies of this system in the pineal gland. In this gland, activation of beta-adrenergic receptors leads to production of melatonin, an effect mediated by production of cyclic AMP. In rats sacrificed at the time of peak withdrawal symptomatology, there was decreased basal AC activity in the pineal gland and a decreased maximal response to guanine nucleotides and isoproterenol. Thus, our preliminary results indicated disrupted function of the beta-adrenergic receptor-coupled adenylate cyclase system in both pineal gland and cortex in two species of animals chronically treated with ethanol by different methods of administration. In both instances, animals were tolerant to and physically dependent on ethanol, and the adaptation of these receptor-coupled AC systems may contribute to certain aspects of behavioral or physiological adaptation. The results described above suggest that, while particular sites of action of ethanol may vary according to the receptor-AC system studied, in general, ethanol enhances the activity of systems in which agonists stimulate AC, and the function of these systems is disrupted after chronic in vivo exposure of animals to ethanol. The changes seen in animals treated chronically with ethanol appear to result in adaptation to the initial effect of ethanol.

In some instances, agonist occupation of receptors results in inhibition of AC activity, and this inhibition appears to be mediated by a class of guanine

nucleotide-binding proteins designated N_i. If ethanol's effects on receptor-coupled AC activity result primarily from alterations in membrane fluidity, ethanol would be expected to alter neurotransmitter-mediated inhibition of AC, as well as its stimulation. In striatum, opiates inhibit AC activity, and in mouse striatal tissue we found that both morphine and [2-D-Ala,5-D-Leu]enkephalin inhibited AC in a dose-dependent manner. However, ethanol, added to the assays in vitro, did not alter the shape of the inhibition curves or the maximal inhibition produced by morphine or enkephalin. Furthermore, when animals were treated chronically with ethanol, there was no difference in the amount or pattern of inhibition of striatal AC produced by morphine or enkephalin. Thus, in contrast to its effects in stimulatory receptor-AC systems, ethanol had no apparent influence on the inhibitory system. These findings suggest that ethanol has a specific site of action at N_s, as opposed to N_i. Whether this specificity is a result of the lipid microdomains surrounding each protein or of the characteristics of the proteins per se is not yet clear.

While a number of neurotransmitter and hormone receptors are coupled to AC, in many instances agonist occupation of receptors results in alterations in ion fluxes rather than activation or inhibition of AC activity. The first step in receptor-mediated alteration of calcium mobilization is believed to be the breakdown of polyphosphoinositide, located in the neuronal cell membrane, to inositol phosphates and diacylglycerol. The coupling factors involved in receptor-mediated polyphosphoinositide (PI) breakdown are not completely elucidated, although recent evidence suggests a role for a guanine nucleotide-binding protein. We measured norepinephrine (NE)- (alpha-1 receptor) and carbachol (CB)- (muscarinic cholinergic receptor) stimulated PI breakdown in mouse brain areas. Ethanol, in vitro, had no effect on NE-stimulated PI breakdown, but ethanol shifted the dose-response curve for CB-stimulated PI breakdown to the right in a dose-dependent manner in cortex, hippocampus, and striatum. Ethanol also inhibited basal (nonstimulated) PI breakdown in all areas. The effects of in vitro ethanol on CB-stimulated PI breakdown appeared to be cumulative during chronic ethanol treatment of animals, and to result in an adaptive change in the acetylcholine response. Thus, at the time of ethanol withdrawal, there was no change in response to NE, but there was a significant shift to the left of the CB dose-response curve in cortical tissue of ethanol-treated animals as compared to controls. We had previously shown that, under identical conditions, there was an increased number of muscarinic cholinergic receptors in mouse cortex, and such an increase in potency of an agonist is characteristic of systems in which an increase in receptor number has occurred. Interestingly, we found no change in the CB dose-response curve in striatal tissue of mice treated chronically with ethanol where we had previously found no change in receptor number. The increased sensitivity to acetylcholine in the cortex may well play a role in the development or expression of certain aspects of ethanol withdrawal symptomatology.

Significance to Biomedical Research and the Program of the Institute:

Overall, our results provide evidence for specific sites of action of ethanol in the CNS, and demonstrate adaptive changes at these sites of action, following chronic ethanol exposure, that may contribute to tolerance and/or physical dependence. The results show that ethanol can selectively modify the function of neurotransmitter receptor-effector coupling processes involved in synaptic transmission in the brain. Our results indicate that if ethanol does act by

perturbing membrane lipids, the nature of various lipid microdomains can lend specificity to the effects of ethanol. Furthermore, since each receptor-effector system responds to ethanol in a specific manner, neurochemical imbalances (i.e., decreased stimulation of AC with no change in inhibition), which contribute to specific signs of ethanol tolerance and/or physical dependence, may occur following chronic ethanol exposure. The demonstration of a functional correlate of a change in neurotransmitter receptor number in ethanol-treated animals is unique, and provides evidence that the alteration in ligand-binding properties is associated with increased physiological sensitivity to agonists. The ability to correlate changes in receptor number with changes in the biochemical consequences of receptor activation should enhance our understanding of the biochemical basis for adaptive physiological and behavioral responses that occur in ethanol-tolerant and ethanol-dependent individuals.

In addition to contributing to our understanding of the mechanism of action of ethanol in the CNS, these studies elucidate some of the basic characteristics of receptor-effector coupling processes. For example, the finding that ethanol does not affect opiate inhibition of striatal AC, but does alter opiate binding (Tabakoff and Hoffman: Life Sci. 32:197-204, 1983; Hoffman, Urwyler, and Tabakoff: J. Pharmacol. Exp. Ther. 222:182-189, 1982) suggests that the mechanisms proposed to explain the actions of N_s and N_i may not apply to opiate modulation of AC activity. Similarly, further studies of the effects of ethanol on ligand binding to beta-adrenergic receptors and on ISO-stimulated AC will enhance understanding of this system. Finally, the fact that ethanol inhibits "basal" PI breakdown suggests that ethanol may also modulate this system at a point distal to the receptor, and provides a possible means for further elucidation of the coupling mechanism involved in this system.

Proposed Course:

Further studies of the effects of chronic ethanol treatment on beta-adrenergic receptor-coupled AC in cerebral cortex and pineal gland are planned in order to define the sites at which adaptive changes occur. These studies will include an investigation of agonist and antagonist binding to beta-adrenergic receptors in the two tissues. If a defect in the activity of N_s is confirmed, studies aimed at characterizing the defect will be initiated. The time course of changes in AC will be correlated with the time course for development and dissipation of tolerance and physical dependence. In vitro organ and tissue culture studies are planned using the pineal gland. Evaluation of the effect of chronic ethanol treatment on receptor-mediated PI turnover in all three brain areas will be completed. Studies of the role of guanine nucleotide-binding proteins in this system, and the effects of ethanol in the presence of guanine nucleotides will be initiated.

Publications:

Saito, T., Lee, J.M., and Tabakoff, B.: Ethanol's effect on cortical adenylate cyclase activity. J. Neurochem. 44:1037-1044, 1985.

Tabakoff, B., Hoffman, P.L., Valverius, P., Borg, S., Lee, J.M., Jaffe, R., U'Prichard, D., and DeLeon-Jones, F.: Characteristics of receptors and enzymes in brains of human alcoholics. Alcohol 2:419-423, 1985.

Hoffman, P.L., and Tabakoff, B.: Ethanol does not modify opiate-mediated inhibition of striatal adenylate cyclase. J. Neurochem. (in press).

Tabakoff, B., and Hoffman, P.L.: Receptor-coupled adenylate cyclase systems in brain: Targets for alcohol action. Substance Abuse (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AA 00703-01 LSN

PERIOD COVERED

November 1, 1984 to September 30, 1985

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

Neurohypophyseal Peptides and Ethanol Tolerance

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

PI:	P. Hoffman	Section Chief	LSN, NIAAA
	B. Tabakoff	Acting Chief	LSN, NIAAA

COOPERATING UNITS *(if any)*

University of Illinois Medical Center (C. Melchior, S. Mannix, B. Cabala, H. Proudfit).

LAB/BRANCH

Laboratory for Studies of Neuroadaptive Processes

SECTION

Section of Neurobiology

INSTITUTE AND LOCATION

NIAAA, 12501 Washington Avenue, Rockville, MD 20852

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.1	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.)*

Arginine vasopressin (AVP), a mammalian antidiuretic hormone, and related peptides, when administered exogenously, prolong the duration of tolerance to ethanol. The similarities between neurohypophyseal peptide effects on tolerance and previously reported effects on memory consolidation supported the hypothesis that, as examples of CNS adaptation, these phenomena may share underlying mechanisms. We found that intracerebroventricular (i.c.v.) injections of AVP and related peptides maintained tolerance in mice, suggesting that the peptides act centrally to modulate neuronal systems influencing tolerance. We have also obtained evidence, in mice and rats, that a specific peptide antagonist, administered i.c.v., can enhance the rate of dissipation of functional tolerance to ethanol. These findings suggest that endogenous AVP may be involved in the maintenance of ethanol tolerance. The effects of AVP on environment-dependent ethanol tolerance have also been studied. This type of tolerance arises as a conditioned compensatory response of the animal to the effects of ethanol, and is more pronounced in an environment in which the animal has previously been exposed to ethanol. Thus, environment-dependent tolerance is thought to have a prominent "learned" component; AVP maintained this tolerance, which was produced by daily injections of ethanol. However, AVP inhibited the acquisition of environment-dependent tolerance, possibly by interfering with the animals' perception of the cues associated with ethanol administration. Preliminary studies of the biochemical effects of AVP in the brain have also been performed. Understanding the role of the peptide hormones in development, expression, and dissipation of various forms of tolerance to ethanol may lead to benign means for the manipulation of tolerance development and, possibly, of ethanol intake.

PROJECT DESCRIPTION:

Investigators:

P. Hoffman	Section Chief	LSN, NIAAA
B. Tabakoff	Acting Chief	LSN, NIAAA
C. Melchior	Asst. Professor	Univ. of Illinois Medical Center
S. Mannix	Graduate Student	Univ. of Illinois Medical Center
B. Cabala	Graduate Student	Univ. of Illinois Medical Center
H. Proudfit	Assoc. Professor	Univ. of Illinois Medical Center

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 exogenous systemic administration of the hormone or its analogs. The goals of this project are to characterize the site of action of AVP for maintenance of tolerance (i.e., central vs. peripheral); to evaluate the role of endogenous hormone in the maintenance of tolerance; to determine peptide effects on acquisition and maintenance of different forms of ethanol tolerance; and to elucidate neurochemical mechanisms by which AVP may influence ethanol tolerance.

Methods Employed:

Male C57Bl mice were implanted with intracerebroventricular (i.c.v.) cannulae, and made tolerant to and physically dependent on ethanol by being fed a liquid diet containing 7% ethanol for 7 days, as previously described (Ritzmann and Tabakoff: J. Pharmacol. Exp. Ther. 199:158-170, 1976). Control mice were pair-fed a diet containing sucrose in amounts equicaloric to the ethanol. On the morning of the eighth day, all animals were given the control diet (withdrawal). At 24 hours after withdrawal, animals were injected i.p. with ethanol (3.1 g/kg), and the duration of loss of righting reflex was measured. This tolerance test demonstrated that all the ethanol-consuming animals were tolerant to the hypnotic effect of ethanol. Control (C) and ethanol-treated (E) mice were then divided into groups that received once-daily i.c.v. injections of artificial CSF vehicle (e.g., C-CSF, E-CSF), or once-daily injections of AVP (1 ng). In some studies, groups of mice were included that received injections of the AVP antagonist, $d(CH_2)_3$TyrOEt-AVP ("Antg," 10 ng) or a combination of AVP and antagonist. All injections were made in a volume of 1.0 μL. When M520 rats were used they were made tolerant to ethanol by exposure to ethanol vapor in an inhalation chamber (Tabakoff and Culp: Alc. Clin. Exptl. Res. 8:495-499, 1984). Controls were in chambers that received air only. In some studies, animals were implanted with intraventricular cannulae before being placed in the chambers. Tolerance was assessed by determining the duration of loss of the aerial righting reflex and blood ethanol level at regain of the righting reflex, after a challenge dose of

2.5 g/kg of ethanol. In studies of the effects of the AVP antagonist on maintenance of tolerance, control and ethanol-treated rats were subdivided into groups that received once-daily i.c.v. injections of $d(CH_2)_5$TyrOEt-VAVP (1.0 μg) or artificial CSF vehicle (all injections were in a volume of 5 μL). Subsets from each group of animals were tested for tolerance at 3 and 9 days after withdrawal.

To induce environment-dependent ethanol tolerance in mice (Melchior and Tabakoff: J. Pharmacol. Exp. Ther. 219:175-180, 1981), the animals were given twice-daily injections of ethanol in a distinct environment. Peptides were administered subcutaneously (40 nmole/kg) or i.c.v. (1 ng) 2 hours following the second ethanol injection, and tolerance to the hypnotic effect of ethanol was assessed following the first ethanol injection. Studies of vasopressin effects on phosphatidylinositol (PI) turnover (polyphosphoinositide breakdown) were performed using slices of mouse hippocampus (Janowsky, Labarca, and Paul: Life Sci. 35:1953-1961, 1984). The slices were incubated with [^{3}H]myoinositol, washed, and then incubated with vasopressin (10^5 M) in the presence of LiCl. Phosphatidylinositol was extracted using $CHCl_3$/MeOH, and the inositol phosphates in the aqueous phase were isolated by column chromatography.

Major Findings:

It has been suggested that AVP and related peptides modify behavior as a result of their aversive properties, which arouse the animal and thereby enhance its ability to learn and/or remember. However, we demonstrated that, in mice, AVP and related peptides administered (i.c.v.) can maintain tolerance to the hypnotic effect of ethanol (Hung, Tabakoff, Melchior, and Hoffman: Eur. J. Pharmacol. 106:645-648, 1984). The dose of AVP used (1 ng/mouse/day) was reported not to affect blood pressure when administered i.c.v., and this same dose was ineffective in maintaining tolerance if administered subcutaneously. These results, together with our earlier work showing that: (1) DGLVP and DGAVP, analogs of AVP with reduced peripheral effects, can maintain tolerance, and (2) intact brain noradrenergic systems are essential for AVP effects on tolerance, strongly support the hypothesis that neurohypophyseal peptides modulate tolerance by acting centrally.

Our earlier studies with peptide analogs demonstrated particular structural requirements for neurohypophyseal peptide maintenance of ethanol tolerance, suggesting that peptide modulation of tolerance may be mediated by brain receptors that recognize AVP. If such receptors are present in the CNS, and resemble peripheral receptors for the hormone, then the actions of AVP on tolerance might be blocked by concurrent i.c.v. administration of specific peptide antagonists and AVP. Similarly, if endogenous AVP is involved in the maintenance of ethanol tolerance, administration of antagonist alone to a tolerant animal would be expected to increase the rate of loss of tolerance. We have performed initial studies of the effects of an AVP antagonist, $d(CH_2)_5$TyrOEt-VAVP, on maintenance of tolerance, and blockade of AVP effects on tolerance. All peptides were administered i.c.v. Treatment of control mice with a mixture of AVP and antagonist did not affect their response to the challenge dose of ethanol; we had previously shown that AVP alone or antagonist alone also had no effect on the response of control mice. At 3 days after withdrawal, ethanol-treated mice receiving CSF or AVP were still tolerant to the hypnotic effect of ethanol as we had previously shown. However, mice that received antagonist alone were no longer tolerant. This dose of antagonist, however, did not appear to block the effect of

exogenously administered AVP. When a higher dose of antagonist (100 ng) was administered alone, we found that the antagonist acted like AVP, and seemed to maintain tolerance. Thus, this antagonist probably has the characteristics of a partial agonist at CNS AVP receptors, as was also reported for peripheral receptors. Nevertheless, the results suggested that endogenous AVP does play a role in the maintenance of ethanol tolerance in mice.

With the ethanol-feeding regimen that we use routinely, mice become tolerant within 5 days, and lose tolerance with a half-life of about 3 days after withdrawal. This time course is reasonable for studying the maintenance, or prolongation, of tolerance by AVP. However, in order to investigate the ability of peptide antagonists to enhance the rate of loss of tolerance, we wished to use a paradigm in which the normal rate of loss of tolerance was slower. Preliminary studies showed that M520 rats developed functional tolerance to ethanol after 5 days in an inhalation chamber, and that tolerance was maintained for up to 12 days after withdrawal. AVP, given subcutaneously, maintained tolerance for 15 days after withdrawal (the longest time tested). M520 rats were then used to assess the effects of the AVP antagonist on the dissipation of tolerance. At 3 days after withdrawal, all ethanol-treated animals were still tolerant. However, at 9 days after withdrawal, the response of ethanol-exposed animals given the AVP antagonist was the same as that of controls, while ethanol-treated animals that received CSF vehicle were still tolerant. The antagonist had no effect on the response of control animals. The blood ethanol level at the time of regain of the righting reflex was significantly higher in the ethanol-treated animals that received CSF than in those that received antagonist. Thus, the antagonist did not simply alter the rate of ethanol metabolism, but enhanced the rate of loss of functional tolerance. (Blood ethanol levels in the two groups of control animals were not significantly different.)

Thus, our results indicate that, in both mice and rats, endogenous AVP may play an important role in the maintenance of ethanol tolerance. Since the AVP antagonist used is thought to block receptor sites for AVP and to act as a partial agonist, these results also support the hypothesis that AVP acts at specific receptor sites in the CNS to maintain tolerance. We have also begun to assess the effects of AVP on environment-dependent tolerance. We and others have shown that, under some circumstances, tolerance to ethanol is a conditioned response. That is, when an animal receives ethanol in a distinct environment, it learns to expect the effect of ethanol in that environment, and demonstrates a conditioned compensatory response to counteract the effect of ethanol. However, this conditioned response is only elicited in the presence of the cues the animal associates with ethanol. If the drug is administered in an unfamiliar environment, tolerance is diminished or absent. Under these circumstances, ethanol tolerance has a significant learned component, and, judging by the effects of neurohypophyseal hormones on learning and memory processes, it was hypothesized that the peptides would substantially modify environment-dependent tolerance. In our laboratories, environment-dependent tolerance could be produced in mice by giving the animals twice-daily injections of ethanol in a distinct environment. In our initial studies, AVP or DGLVP, administered s.c., inhibited the rate of extinction of environment-dependent tolerance, similar to results seen in studies of memory, and also similar to our results using the liquid diet method to induce tolerance (environment-independent tolerance). However, somewhat surprisingly, we found that the peptides decreased the rate of acquisition of tolerance. It seemed

possible that the peripheral, aversive affects of the peptides might seriously interfere with the conditioning procedure. More recently, we have administered AVP by the i.c.v. route, both during and after the period of ethanol treatment. Our results show that AVP reduced both the rate of acquisition and the rate of loss of ethanol tolerance. The decreased rate of loss of tolerance is, again, consistent with our previous results on tolerance, and with the effects of AVP on memory processes. The reduced rate of acquisition of tolerance seems inconsistent with the effects of AVP on learning in general, but it is possible that the administration of AVP may interfere with the animal's perception of the cues associated with ethanol administration.

Assuming that AVP interacts with a specific receptor in the CNS in order to exert its effects on tolerance, it should be possible to elucidate the biochemical response to occupation of this receptor. Peripheral AVP receptors have been shown to be coupled either to adenylate cyclase activity or to stimulation of phosphoinositide (PI) breakdown. A recent report demonstrated specific AVP binding sites in hippocampus, and we therefore investigated the ability of AVP to increase hippocampal PI breakdown. We found only a small effect of AVP in this system, and further studies are needed to evaluate the importance of this possible mechanism of AVP action.

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, can modulate tolerance provides the opportunity for development of benign therapies to modify tolerance development. More theoretically, 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. Such studies also contribute to the understanding of the biochemical mechanisms underlying learning and memory.

Proposed Course:

We will characterize in more detail (e.g., dose response, site of action) the ability of AVP antagonists to enhance the rate of loss of ethanol tolerance. We will also investigate the dose ratio of AVP to antagonist, which is necessary in order for antagonist to block the effects of AVP on tolerance. In the studies of environment-dependent tolerance we will investigate peptide effects on the rate of extinction of tolerance, as opposed to its natural rate of loss. We plan to begin to evaluate in more detail the CNS site of action of AVP for maintenance of tolerance by performing site-specific injections. These studies will complement biochemical analyses of AVP binding in various brain areas and peptide effects on adenylate cyclase activity and PI breakdown. In all cases, analogs that have been tested for effects on tolerance will also be used for biochemical studies in order to correlate biochemical and behavioral properties.

Publications:

Colbern, D.B., Haaf, J.T., Tabakoff, B., and Greidanus, T.B.: Ethanol increases vasopressin shortly after intraperitoneal injection in rats. Life Sciences (in press).

Hoffman, P.L.: Central Nervous System effects of Neurohypophyseal Peptides. In: Smith, C.W. (Ed.): The Peptides. Chemistry & Biology of Neurohypophyseal Hormones. New York, Academic Press (in press), vol. 8.

INDEX

ANNUAL REPORT

October 1, 1984 - September 30, 1985

* Includes all projects active in FY 1984 and FY 1985, some of which have been consolidated or terminated during FY 1985. See individual project reports for status.

70390
JL

http://nihlibrary.nih.gov

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

NIH LIBRARY
3 1496 00427 5601